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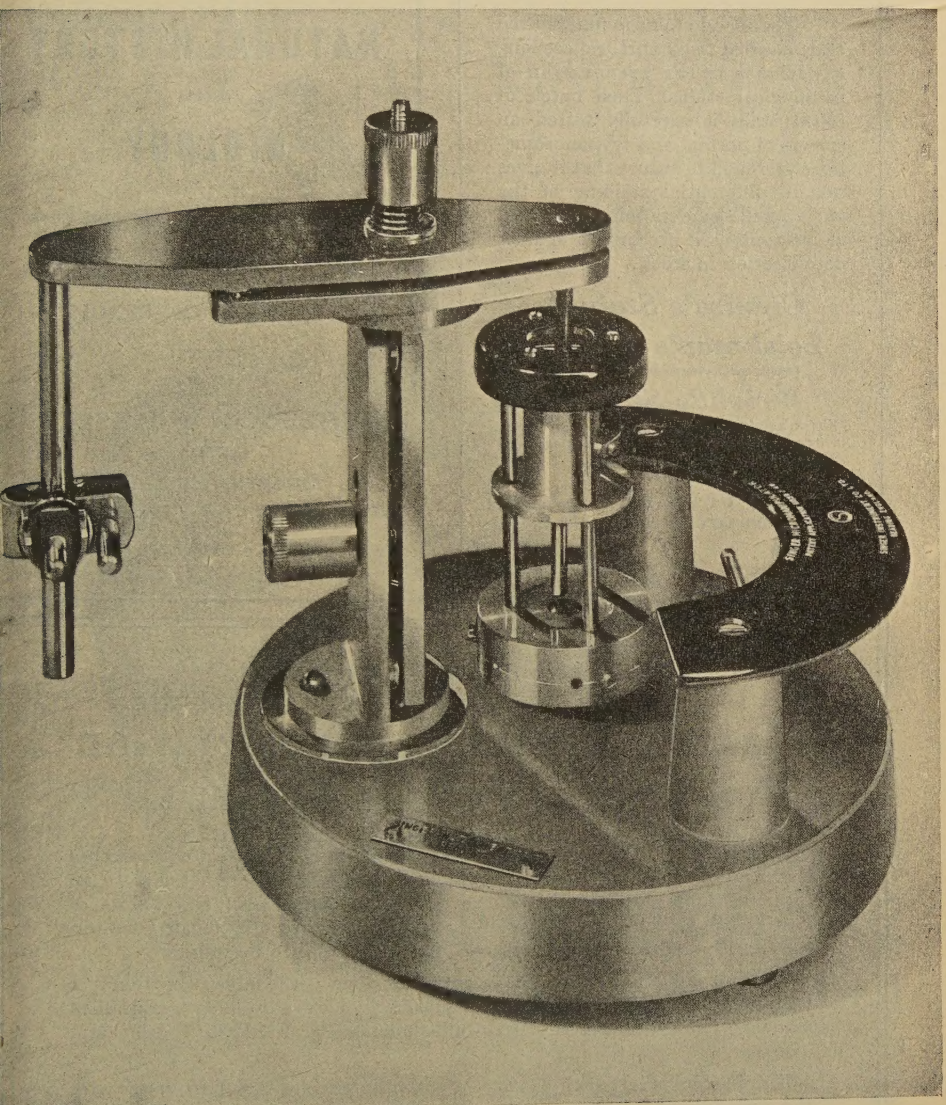

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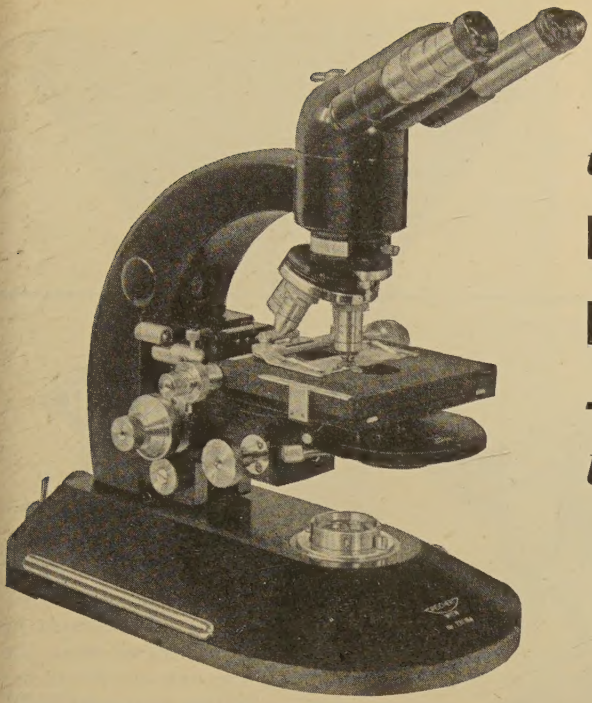

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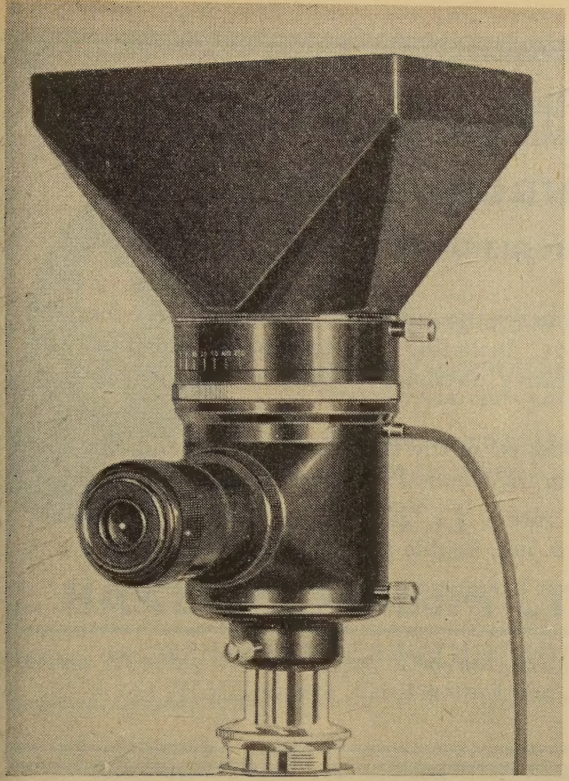

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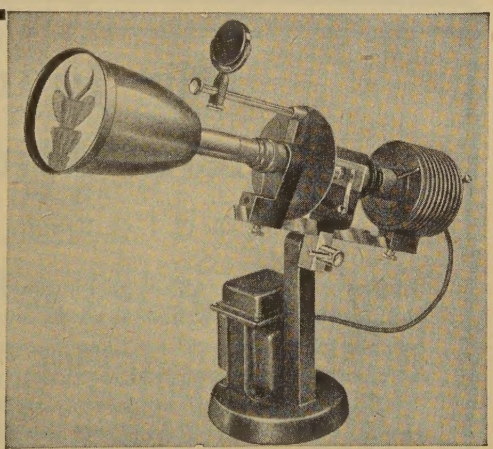

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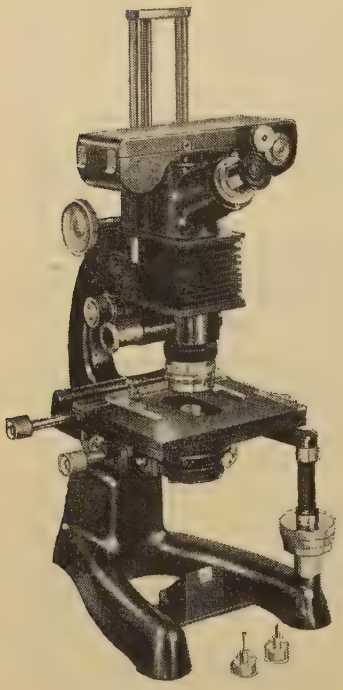

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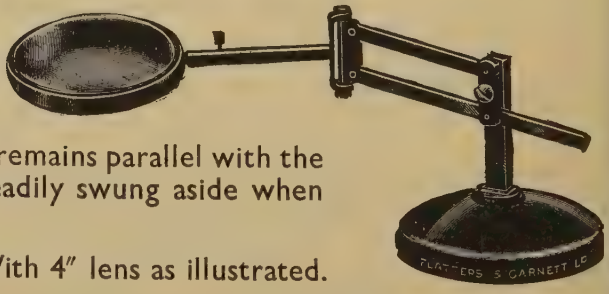

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A Simple Apparatus for Freezing-Drying Histological Specimens

By H. KRAMER, M.B., Ch.B., D.Phil., and R. G. HILL, F.I.M.L.T.

(*From the Dept. of Experimental Pathology, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. Dr. Kramer's present address is Special Unit for Investigation and Treatment of Cancer, Prince of Wales Hospital, Sydney, N.S.W., Australia*)

Summary

The construction and operation of a simple apparatus for freezing-drying histological specimens is described. The apparatus, which is constructed from materials readily available in most laboratories, requires neither elaborate pumping equipment nor vapour traps, for the water molecules emanating from the specimens are carried away by a stream of dry gas. By this means specimens can be dried from a lower temperature in a time considerably shorter than is possible with the more complicated and costly freezing-drying apparatus usually used.

MANY of the conventional types of freezing-drying apparatus employ an oil or mercury diffusion-pump, backed by a rotary pump, to obtain the high vacua necessary to ensure that the water molecules which escape from the specimens have sufficient mean free path to enable them to reach the vapour trap. Clearly, the efficiency of the freezing-drying process might be greatly increased if the specimens could be placed inside a diffusion-pump designed to operate at the necessary low temperature. In such an apparatus the water molecules which escape from the specimens would be entrained in a stream of gas and carried out of the system. As considerations of mean free path would then become unimportant, the need for vapour traps and ultra-high vacua would be eliminated.

The first steps towards the design of an apparatus of this sort were taken independently by Treffenberg (1953) and Jensen (1954), both of whom effected dehydration of specimens by means of a stream of dry air. The devices designed by both these authors involve elaborate measures for drying the air and maintaining the necessary low temperature of the air and specimens during the drying process. The apparatus about to be described here is greatly simplified in that it uses solid carbon dioxide as the source of both dry gas and refrigeration. Any moisture which might be present in the carbon dioxide snow is effectively trapped, so that for practical purposes the gas evolved can be regarded as dry.

Construction and Assembly of the Apparatus

The essential components of the apparatus are shown in figs. 1 and 2.

A mixture of carbon dioxide snow and cellosolve (ethylene glycol monoethyl ether), contained in a wide-mouth household vacuum flask of about one litre capacity, serves to cool the cryostat and provide a steady stream of dry gas. A simple safety-valve which will prevent any building up of pressure in

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the container, and at the same time exclude atmospheric air, is provided by a motor-tire valve mounted in a glass tube passing through the bung.

The cryostat (fig. 1) is made from a metal Seitz-type bacteriological filter-funnel. In place of the asbestos filter-pad, a rubber washer or 'O' ring is inserted between the upper and lower components. A disk of tough (Whatman No. 5) filter-paper is cut to fit snugly beneath the perforated metal plate which normally supports the filter-pad. The throat and upper part of the stem of the funnel are tightly packed with Kieselguhr, which is tamped down with a plug of cotton wool. The tightness of this choke determines the pressure at which

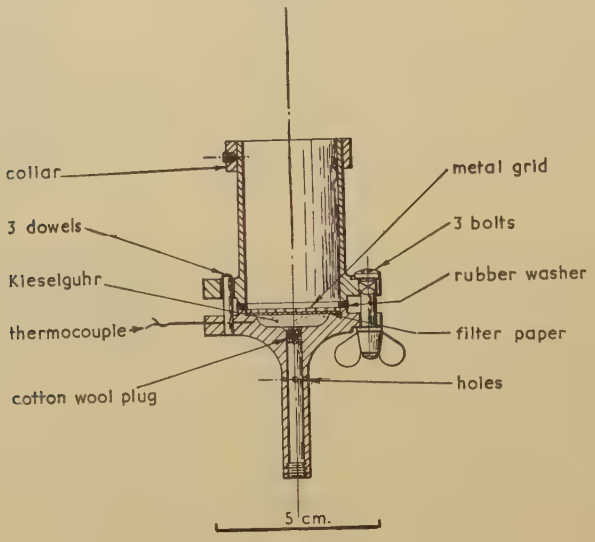

FIG. 1

the apparatus operates. (It is convenient to pack this choke to the desired tightness while the cryostat is connected through a manometer to the pump.) Several holes are drilled in the stem of the funnel, below the choke, to admit gas. Cooling of the cryostat is achieved by means of a heat exchanger, consisting of a length of copper rod $\frac{3}{16}$ inch in diameter, one end of which is threaded to screw into the stem of the funnel, while the other dips down into the refrigerant.

The cryostat is mounted in the bung of the vacuum flask as shown in fig. 2. The cork usually provided with the flask will serve if it is rendered air-tight by impregnating it with paraffin wax in a vacuum. If the cork stopper is used it is as well to secure the cryostat by means of a metal collar retained by two grub-screws, round the protruding portion. If a rubber stopper of the required size is available it is, of course, preferable. In practice, the cork stopper is quite satisfactory, provided that all actual and potential inward leaks are sealed with silicone vacuum grease. Such leaks are easily detected by the deposition of frost on the underside of the cork.

The top of the cryostat is closed by a rubber stopper through which passes

a length of glass tubing, connected to the pump (Edwards Speedivac Single Stage Model 1. SP. 30), through a manometer, by means of rubber pressure-tubing. The glass tube passes through a waxed cork, in the lower side of which a recess is machined so that it fits closely over the cryostat. This cork can be pushed down when the apparatus is in operation, and can be raised when it is necessary to open the cryostat for introducing or removing specimens. It

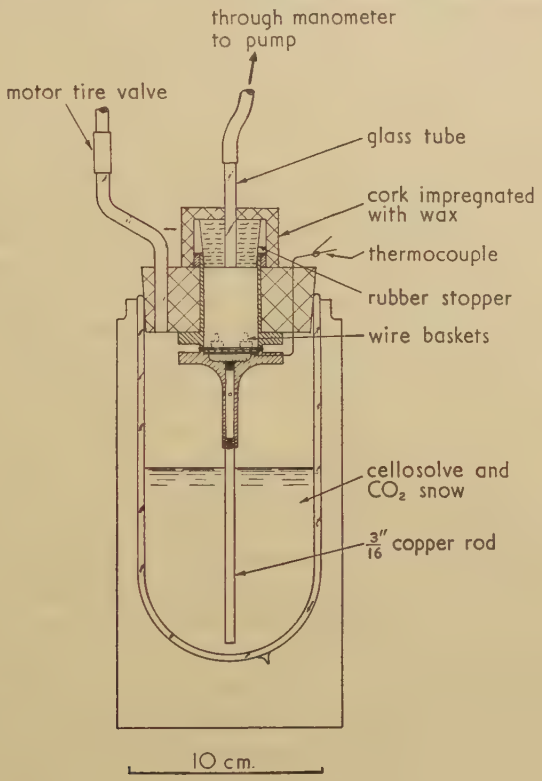

FIG. 2

serves to provide thermal insulation and to prevent frosting of the protruding portion of the cryostat.

The temperature of the cryostat during operation is measured by a copper-constantan thermocouple cemented into a hole drilled in the lower portion close to the metal grid on which the specimen-containers rest.

It is convenient to place the specimens in small copper gauze baskets, about a centimetre or less in diameter.

Operation of the Apparatus

The vacuum flask is about half-filled with carbon dioxide snow, into which 100 to 150 cc. of cellosolve is stirred to make a sludge. The cellosolve serves to maintain even contact between the heat exchanger and the refrigerant. The

depth of the dry ice/cellosolve mixture determines the temperature at which the apparatus operates. This has to be determined empirically in the first instance and adjusted accordingly. Having done this it is advisable to mark the level for future use. The apparatus is now assembled as illustrated in fig. 2, the pump started, and the cryostat allowed to cool down for about half an hour before the specimens are introduced.

The specimens, in their copper-gauze baskets, are refrigerated in isopentane cooled by liquid nitrogen in the usual way. The pump is stopped and a few minutes are allowed for the pressure in the cryostat to rise. The cryostat is then opened, the specimen containers introduced, the stopper replaced and the pump restarted.

As carbon dioxide is consumed the level of the dry ice/cellosolve mixture in the flask falls, so that the temperature of the cryostat rises gradually. Over a period of 24 hours, with an ambient temperature in the vicinity of 30° C, the temperature usually rises some 8° to 10° C. It is therefore advisable to begin the drying process at a temperature lower by that amount than the maximum temperature which one wishes the specimens to attain. In practice it appears safe to begin drying at about −45° C, so that during a 24-hour run the temperature of the specimens never rises above −35° C.

Discussion

Although the apparatus described differs in many respects from the oil or mercury diffusion-pumps used in high vacuum practice, the underlying principle is not greatly different. Both depend on the unwanted molecules—in this case water, in high-vacuum work gas—being entrained and carried along by molecular bombardment in a stream of vapour generated by boiling the working substance. In the conventional diffusion-pumps the vapour, after it has served to carry the unwanted gas out of the system, is condensed and returned for re-use; in this apparatus the carbon dioxide is pumped out of the system together with the entrained water molecules. A similar principle is employed in low-pressure distillation of liquids of high boiling-point, where a stream of air, entering the distillation flask through a controlled leak, assists the movement of vapour to the condenser.

Limitations imposed by considerations of the mean free path of the water molecules emanating from the specimens do not arise in an apparatus of this sort. There is no question of the water molecules finding their own way to the vapour traps; instead, they are actively swept away by the stream of carbon dioxide passing over the specimens.

The pressure at which the apparatus should operate for maximum efficiency remains to be determined. Satisfactory results are obtained at a pressure of 2–3 mm of mercury. This figure was arrived at empirically. It represents a compromise between encouraging the sublimation of ice at reduced pressure, passing enough gas over the specimens, and at the same time not depleting the supply of carbon dioxide snow too rapidly and so increasing the rate at which the temperature of the cryostat rises. With pressures of about 3 mm of

mercury the pumping rate is found to be about 44 ml of carbon dioxide per minute (corrected to N.T.P.). This represents a consumption of about 120 g of dry ice in 24 hours.

The gradual rise in temperature which occurs during the drying process constitutes no serious disadvantage, as the temperature is lowest whilst water is subliming from the surface layers of the specimen, and is rising during the period when the resistance of the dried shell is becoming increasingly important as a factor limiting the rate of escape of water molecules.

The apparatus has been extensively used for some months and its performance is highly satisfactory. In general, 18 hours suffices for thorough drying of blocks of tissue of the size customarily used with freezing-drying techniques, although for the more solid tissues, such as liver and kidney, as well as fatty tissues, 24 hours is perhaps safer. In the case of lung, intestinal mucosa, and skin as short a period as 10 to 12 hours is usually adequate. The adequacy of drying has been assessed by weighing the specimens accurately before and after dehydration, and comparing the percentage weight loss with the known water-content of the tissues; and also, by observing the quality of histological preparations obtained after varying periods of drying.

The apparatus is constructed from materials readily available in most laboratories and can be made in a few hours. Even the mechanical pump is not essential, for if a suitably steady mains pressure is available a simple water pump is adequate. Clearly many refinements are possible, the most obvious being the introduction of a thermostat to control the temperature of the cryostat. However, the apparatus as it stands performs perfectly adequately; its simplicity and efficiency should lead to the more widespread use of frozen-dried material in cytological work.

It is a pleasure to acknowledge the assistance given by Mr. J. S. Coombs in making the equipment for measuring temperature, and the many helpful suggestions of Dr. G. B. Mackaness.

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Preservation of Animal Tissues, with a Note on Staining Solutions

By G. OWEN AND H. F. STEEDMAN

(*From the Department of Zoology, The University of Glasgow*)

SUMMARY

Methods are given by which animal material, fixed by any desired method, may be preserved against attack by fungi or bacteria. The reagents employed are glycol monophenyl ethers ('Phenoxetol'), and esters of *p*-hydroxybenzoic acid ('Nipa' esters). These reagents, dissolved in water, produce colourless and almost odourless solutions, in which a variety of animals have been preserved for many months without showing any traces of deterioration or decomposition.

PRESERVATION of animal tissues is usually undertaken with one of three ends in view: for dissection, for museum display, or for histology. Fixation of tissues differs from preservation in that the former is designed to render most or some special elements insoluble and proof against further change. The aim of preservation is to maintain this condition.

Some reagents such as 75% methylated spirits in water, and 10% formalin may serve both functions, but as preserving fluids they have the disadvantage that they are volatile, and formaldehyde is pungent and unpleasant to use. An ideal preserving fluid should have the following properties. It should

1. maintain the fixed tissues in good condition, and
2. be pleasant to handle,
3. odourless,
4. non-volatile,
5. transparent,
6. colourless,
7. non-poisonous,
8. bactericidal and fungicidal,
9. low in price, and
10. maintain the natural colour of the specimen.

Ethylene glycol monophenyl ether and *p*-hydroxybenzoic acid, with their derivatives, possess most of these requirements. They are manufactured by Nipa Laboratories, Treforest, Pontypridd, Glam., the former compounds being referred to as 'phenoxetols', the latter as 'Nipa' esters. In their pure forms these compounds may be considered poisonous, but in the dilutions suggested here they may be used freely and without special precautions. The bacteriostatic and bactericidal value of ethylene glycol monophenyl ether was reported by Berry (1944). The narcotic properties of phenoxetol (Gough and others, 1944), and of propylene phenoxetol (Owen, 1955) have been mentioned, also the preserving properties of propylene phenoxetol used with gelatine solutions (Gough and Wentworth, 1949). The capacity of propylene phenoxetol to

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restore the natural colour to blood-pigments in pathological specimens was commented on by Gough and Wentworth (1949), and the value of the esters of *p*-hydroxybenzoic acid as preservatives in ointments, &c., is generally recognized (Boehm, 1933).

Preliminary experiments on the preservation of animal tissues by propylene phenoxetol and Nipa ester combination No. 82121 have been carried out in this Department. It is regretted that none of the animals has been preserved for longer than 6 months, but the method is considered so promising that an early account was considered desirable. With propylene phenoxetol as a preserving fluid the procedure is as follows:

1. Fix the specimen for the minimum length of time (12 hours for an opened frog; 48 hours for an opened dogfish) in 10% formalin or 70% alcohol. Frogs and similar vertebrates should be fixed in a position suitable for subsequent dissection. Improved colour-retention of the specimens may be obtained by fixing in the following solution (Gough and Wentworth, 1949):

Formaldehyde, 40%	500 ml
Sodium acetate	500 g
Water	5,000 ml

2. Wash in running water 12 to 24 hours.
3. Transfer the specimen to 1% propylene phenoxetol in water.

The procedure with 'Nipa' ester combination No. 82121 is essentially the same. Transfer the specimens after fixation and washing to 0·2% Nipa ester in water. As the rate of solution of both propylene phenoxetol and Nipa ester is rather slow, it is essential to add them slowly to water and to stir vigorously at the same time, or alternatively to keep stock solutions of 20 ml of propylene phenoxetol or 20 g of Nipa ester in 80 ml of industrial methylated spirits. The required volume of stock solution is then added to water with stirring. The alcohol aids the dispersion and solution of the preservatives. If only a small volume of preserving fluid is required, propylene phenoxetol or Nipa esters may be added to warm or boiling water and the solution allowed to cool, but for large containers such as dogfish tanks the alcoholic solution method is to be preferred.

The animals which have been preserved in the above solutions are as follows:

Animal	*Fixative*	*Preservative*
Acanthias vulgaris	Formalin	0·2% Nipa ester and 1% propylene phenoxetol
Bufo bufo	Formalin	1% propylene phenoxetol
Rana temporaria	Formalin	1% propylene phenoxetol
Mus musculus	Formalin	1% propylene phenoxetol
Sheep heart	Formalin	1% propylene phenoxetol
Helix pomatia	Formalin	1% propylene phenoxetol and 0·2% Nipa ester
Various lamellibranchs	Alcohol and formalin	1% propylene phenoxetol and 0·2% Nipa ester

After 6 months all the specimens were of excellent texture and appearance, with no trace of mould or bacteria—excellent for dissection or museum display. The frog, toad, and sheep heart retained some of the natural colour of the blood.

The following comparative prices per litre of diluted preserving fluids may be of interest.

10% formalin	3·8*d.*
10% perminal KB (Steedman, 1955)	4·0*d.*
75% methylated spirits	10·0*d.*
0·2% Nipa ester No. 82121	1·2*d.*
1% propylene phenoxetol in water	15·7*d.*

It should be emphasized strongly that these solutions are preserving solutions, to be used only after adequate fixation. Animals such as fresh dogfish placed directly in the above solutions will decompose.

The Preservation of Dye Solutions

The aqueous solutions of many commonly used stains develop moulds and frequent filtration is necessary. Moreover, the intensity of the staining reaction decreases with time and it is advisable to use freshly made solutions. The addition of thymol merely reduces the rate of growth of the mould, or at higher concentrations it may produce an undesirable deposit. These disadvantages may be overcome by dissolving the stain either in 0·5% propylene phenoxetol in water or in 0·1% *p*-chlorophenoxetol (the *p*-chloro-phenylether of ethylene glycol) in water. The compounds should be added to warm water (50 to 60° C), and the stain added when the water has cooled to room temperature.

This method produces mould-free staining solutions of aqueous eosin, alcian blue, &c., which are as vigorous in their staining reactions after 6 months as they were when fresh.

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Human Saliva as a Convenient Source of Ribonuclease

By S. BRADBURY

(*From the Cytological Laboratory, Department of Zoology, University Museum, Oxford*)

Summary

Saliva, heated to 80° C for 10 minutes and centrifuged to remove mucus, is used as a source of ribonuclease. Slides of mouse pancreas incubated in this for 3 hours at 60° C show complete removal of cytoplasmic basiphilia. Incubation of control slides in distilled water before staining is essential in order to avoid errors due to possible solution of basiphil material in aqueous media. Errors due to incomplete elimination of the salivary amylase are prevented by using the PAS reaction to check the absence of all diastatic activity.

Formaldehyde and phenyl isocyanate are used as specific enzyme inhibitors. If these are added to the saliva before use, the ribonuclease is inactivated, and there is then scarcely any action on the cytoplasmic basiphilia.

With these controls, incubation of slides in prepared saliva, followed by staining with basic dyes, may be used in histochemistry as a means of localizing ribonucleic acid.

Introduction

THE enzyme ribonuclease, in conjunction with staining by basic dyes, was used by Brachet (1953) as a means for the cytochemical detection of ribonucleic acid. It is customary in such work to use crystalline ribonuclease, as prepared by Kunitz (1940). One of the chief criticisms of the use of such enzyme preparations is that traces of proteolytic activity may be present, though McDonald (1948) has succeeded in obtaining the crystalline enzyme free from all proteolytic activity. The complex method of preparation is a drawback to the use of this extremely pure enzyme in general histochemical practice. Pearse (1954) draws attention to the fact that a preparation of ribonuclease, free from protease and amylase, may be obtained by heating human saliva for short periods. It is the purpose of this paper to give practical details of the preparation and use of this convenient source of ribonuclease.

Materials

Seven micron paraffin sections of mouse pancreas fixed in Zenker's fluid for 3 hours were used as a standard test object. In addition, transverse sections of the leech *Glossiphonia complanata* fixed in neutral 40% formalin were used in certain of the experiments.

Method

Preparation of the enzyme source

1. Collect about 40 ml. of saliva.
2. Place in a cool water bath and heat until the saliva reaches a temperature of 80° C. Keep the saliva at this temperature for 10 minutes.
3. When cool, centrifuge for 5 minutes in order to remove most of the mucus. The supernatant, which should be an opalescent, rather viscous liquid, is used as the enzyme source.

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4. Put the treated saliva in a corked specimen tube which is large enough to hold a microscope slide.

It is advisable to use the saliva as soon after preparation as possible.

Use of the treated saliva to remove cytoplasmic basiphilia.

1. Bring paraffin sections of the material to water. The best results are obtained with tissues fixed for a short time in Zenker's fluid or in formaldehyde.
2. Incubate slides in the saliva for 3 hours at a temperature of 60° C.
3. Wash in running water for 1 minute.
4. Dry round the section with a cloth. Stain in pyronin / methyl green for 30 minutes.
5. Rinse in distilled water.
6. Blot dry; absolute acetone, 1 minute; acetone-xylene, 1 minute; mount in DPX.

The pyronin / methyl green is prepared according to the instructions given by Jordan and Baker (1955).

Result

Basiphilia of the cytoplasm and of the nucleolus is indicated by coloration with pyronin. If present in the control slides but absent in slides treated with the saliva before straining, the basiphilia is due to the presence of ribonucleic acid.

Discussion

Erroneous results may be obtained for the following reasons:

1. solution of the basiphil material in the aqueous incubation medium;
2. the possibility that the enzyme acting on the cytoplasmic basiphilia is not ribonuclease, owing to incomplete elimination of the salivary amylase.

Solution of basiphil material in the aqueous incubation medium is the most likely source of error (Lison, 1953), especially as in this case the incubation is carried out at a rather high temperature. The optimum temperature of ribonuclease is 70° C. (Dubos and Thompson, 1938). Since saliva has such a low concentration of this enzyme, a temperature near the optimum was chosen, so as to avoid long periods of incubation. As a control, it is suggested that a slide should be incubated in glass-distilled water at the same temperature as the test slides. If there is any appreciable reduction of the cytoplasmic basiphilia after this treatment, no conclusions may be drawn as to the nature of the basiphil material.

With the tissue used in the present experiments, namely pancreas of the mouse, an incubation period of 18 hours in distilled water did not remove any basiphilia from the exocrine cells. Consequently it may be assumed that in the case of this particular material and fixative, no error is being introduced through solution of any basiphil tissue-component in aqueous media.

The second source of error may be that the active principle of the prepared

saliva is not in fact ribonuclease. It is a well-known fact that other enzymes are present in saliva in addition to salivary amylase and ribonuclease. Most, however, are present in such minute amounts that there is little chance of their affecting the cytoplasmic basiphilia.

Though Kunitz (1940) in his study of ribonuclease pointed out the great stability of this enzyme, it was thought necessary to attempt its inhibition by heat and specific inhibitors. In one experiment, a sample of saliva was boiled for 5 minutes before use. Slides stained after incubation in this preparation showed little basiphilia in the exocrine cells; it was concluded, therefore, that although there had been some slight inactivation by heat, this process was by no means complete. A similar experiment, with saliva made slightly alkaline by 1% NaOH before boiling, showed that there was no more destruction of enzyme than when the saliva was heated alone. These results agree with the findings of Kunitz, who showed that ribonuclease is only gradually destroyed by heating in an acid medium, and is only slowly inactivated by heating in a medium of pH 12 or higher.

Zittle (1948) points out that ribonuclease can be inhibited by reagents such as formaldehyde and phenyl isocyanate, which react with amino groups. With formaldehyde, the inhibition of the enzyme is not quite complete; with phenyl isocyanate, the most specific inhibitor for ribonuclease at present available, almost complete inhibition can result. Both reagents were tested with saliva prepared in the usual way. Five millilitres of neutral 40% formalin were added to 40 ml. of the saliva, and slides incubated and stained as before. Control slides were treated with similar amounts of formalin and distilled water. In the experimental series there was a very slight reduction of the basiphilia in the cells at the centre of the block. In the controls no change in basiphilia was noticed, though in both cases the action of the formalin altered the staining of the nuclei.

In a further experiment, 0·5 ml of phenyl isocyanate was added to the saliva, which was then allowed to stand for 5 minutes with occasional shaking before recentrifuging in order to remove a white precipitate which had formed. It was found that in slides incubated in this mixture there was a very slight reduction of the cytoplasmic basiphilia, which was not apparent in the control slides. These experiments with formaldehyde and phenyl isocyanate show that there had been a very marked inhibition of the enzyme. The inhibition was not quite complete, but it was sufficiently marked to leave little doubt that the active principle removing the cytoplasmic basiphilia was ribonuclease.

As a further test of the saliva used, it was thought worth while to confirm that all the diastatic activity had been destroyed by the heat treatment. This was done by carrying out the periodic acid / Schiff reaction on sections of frog liver fixed in Rossman's fluid; in untreated sections, the glycogen gave a strong positive reaction to the test. Further sections were incubated for 1 hour at 60° C in the saliva and in distilled water before doing the test. In both cases, the reaction was just as strong as in the untreated control slides, so that it may be concluded that the heat treatment of the saliva completely inactivates the

salivary amylase. It is desirable to do this test on every saliva preparation before using it as a source of ribonuclease; if any indication of diastatic activity is obtained, then the preparation should be heated until all such activity is destroyed.

As an alternative to the PAS test, the starch-iodine reaction may be used. Two millilitres of a 5% solution of soluble starch is mixed with 1 ml of the prepared saliva. One drop of the mixture is immediately removed, placed on a white tile, and tested with 1 drop of dilute iodine solution; a strong blue colour should develop. Further drops are removed at intervals and tested with the iodine. If the salivary amylase has been completely inactivated, then no digestion of the starch will take place and the blue colour will be obtained even in samples taken after 15 minutes. In a control experiment with untreated saliva all traces of starch disappeared after 3 minutes.

Studies were made of the time of incubation required to remove the cytoplasmic basiphilia in mouse pancreatic tissue. It was found that even after such a short time as 15 minutes, a considerable reduction in basiphilia had occurred. Complete elimination did not take place until incubation in the enzyme had been prolonged to 3 hours.

The cells at the edge of the block appeared much more resistant to the action of the enzyme, retaining their basiphilia long after the cells in the centre of the tissue. In these external cells, the zymogen granules were noticed to be strongly pyroninophil, even after the 3-hour incubation in the enzyme which suppressed basiphilia in all other cells. These effects could be due to some action of the fixative; the difference between the centre of the block and its edges may be explained as a consequence of differing rates of penetration of the components of the fixative. Two other interesting features were noticed: the red blood-cells, after incubation in the enzyme, became strongly stained with pyronin, and the nuclear staining changed from a bluish-green to a pure light green. This latter observation agrees with the views of numerous other workers that the nucleus in addition to the DNA contains ribonucleic acid. In the slides stained with pyronin / methyl green, this would take up some of the pyronin so that the resultant mixture of red and green would give the observed bluish appearance. The enzyme ribonuclease removes this RNA from the nuclei, so that when they are stained with pyronin / methyl green after this treatment, they only take up the methyl green and appear much lighter in colour. If the period of incubation was prolonged to 12 hours, the nuclei lost their capacity to stain with this dye. A similar effect, studied by Pollister and Leuchtenberger (1949), was shown to be due almost entirely to the depolymerization of the nucleic acid. It is probable that the hot aqueous incubation medium used in the present experiments is having a similar effect. At the moment no explanation can be put forward for the marked affinity of the red blood-cells for pyronin after incubation in the saliva preparation; possibly it is due to some alteration in the cell-wall.

As a result of these experiments, it seems that the ribonuclease from saliva may be used in histochemical work if the following precautions are taken.

1. Control slides must be carried through comparable incubations in glass-distilled water, and preferably through saliva which has had the ribonuclease inactivated in some way.
2. Control slides must be used to check that the inactivation of the salivary amylase is complete.
3. The cells at the extreme edges of the block should be disregarded when evaluating the results.

I wish to thank Dr. J. R. Baker for suggesting the possibility of using saliva as a source of ribonuclease, and for helpful advice and encouragement throughout the work. My thanks are due also to Prof. A. C. Hardy, F.R.S., in whose department I am working, and to Mr. A. R. Fontaine for kindly repeating some of the experiments. This work was carried out during the tenure of a Senior Hulme scholarship of Brasenose College, Oxford, and a grant from the Department of Scientific and Industrial Research.

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Mucopolysaccharides and the Calcification of the Scale of the Roach (*Leuciscus rutilus*)

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With one plate (fig. 1)

Summary

1. The scale of the roach (*Leuciscus rutilus* L.) was examined for state of calcification, metachromasy, and reaction to the periodic acid / Schiff (PAS) test.

2. Metachromasy and a positive PAS-reaction imply acid mucopolysaccharides in the bony layer.

3. There is a great increase in these reactions and in reactions for bone salts in the transition zone between the uncalcified and calcified part of the bony layer.

4. These reactions imply that the bond between the osseoid and the inorganic fraction of the bony layer is through SO_2- and PO_4-groupings.

5. The fibrillary plate lacks metachromasy, but shows a positive PAS-reaction. Before calcification a strong orthochromasy points to acid groups in connexion with the collagen. Under the radii there is no orthochromasy, and the PAS-reaction is negative.

6. When calcifying, the fibrillary plate loses its orthochromasy and the mineral matter is deposited as large crystal-complexes.

7. In regenerating scales the reactions are weaker than in normal scales.

Introduction

THIS investigation deals with the occurrence of mucopolysaccharides and with the calcification of fish-scales as exemplified by the scales of the roach, *Leuciscus rutilus L.* It forms a part of an investigation of the growth of the fish-scale.

Mucopolysaccharides in the form of chondroitin sulphuric acid (CSA), have been identified in most normal calcified tissues, such as dentine and enamel (Pincus, 1950; Bevelander and Johnson, 1955), bone (Logan, 1935; Hass, 1943; Sylvén, 1947), and tendon (Rubin and Howard, 1950), and also in pathologically calcified arteries (Faber, 1949).

It was suggested by Logan (1935), Hass (1943), and Sylvén (1947) that CSA is removed from cartilage before the calcification of the latter takes place. However, Rubin and Howard (1950) showed that after decalcification metachromasy reappears, which indicates that CSA is left in the tissue. Metachromasy is weakened or removed *in vitro* also by Ca-ions (Levine and Schubert, 1952). Correspondingly it has been shown that synthetic CSA-collagen complexes behave like rachitic cartilage as regards metachromasy and capacity for calcification (Sobel and Burger, 1954).

A positive reaction to Hotchkiss's periodic acid / Schiff (PAS) test has been reported in most calcified tissues, such as bone (Rubin and Howard, 1950; Bevelander and Johnson, 1950; Heller-Steinberg, 1951; Pritchard, 1952) enamel, and dentine (Bevelander and Johnson, 1955).

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Material and Methods

Scales from roaches, both normal and after regeneration for 7 days at 20° C. have been used. For the identification of metachromatic substances the sections have been treated with toluidine blue according to Lison-Sylvén (Glick, 1949). Mucopolysaccharides have usually been identified by Hotchkiss's PAS method (Glick, 1949), in a few cases according to Bauer-Feulgen (Glick, 1949). The lime salts have been made visible by the methods of Heller-Steinberg (1951) and Stoeltzner (1905).

Observations

The fish-scale is composed of two layers: the bony layer and the fibrillary plate. The homogeneous bony layer on the external side of the scale carries on its surface more or less concentrically arranged ridges or striae. The fibrillary plate under the bony layer is built up of thin lamellae, composed of parallel fibres. The bony layer is absent from the radii, which thus are furrows running in the direction from the focus to the periphery (fig. 1, A). Under the radii the fibrillary plate has a different chemical composition (fig. 1, F).

My observations regarding ortho- and metachromasy, PAS-reaction, and state of calcification are assembled in table 1.

Discussion

It appears from table 1 that the two distinct parts of the fish-scale, the bony layer and the fibrillary plate, differ with regard to the polysaccharides and mode of calcification.

The reactions of the bony layer agree well on the whole with those of bones and teeth. Metachromasy and a positive PAS-reaction, occurring simultaneously, give strong evidence for acid mucopolysaccharides: in fact, all known acid mucopolysaccharides give both reactions (Rubin and Howard, 1950). These reactions suggest the presence of CSA, in cartilage, bone, and teeth, although as far as the author is aware this substance has not been isolated from scales.

Fig. 1 (plate). A, the edge of a roach-scale in whole mount, stained after Stoeltzner. *a*, the pale osseoid, followed inwards by *b*, the deeply coloured zone of calcification, and *c* the more weakly stained bony layer with *d*, a stria. A radius *e* causes an interruption in the bony layer.

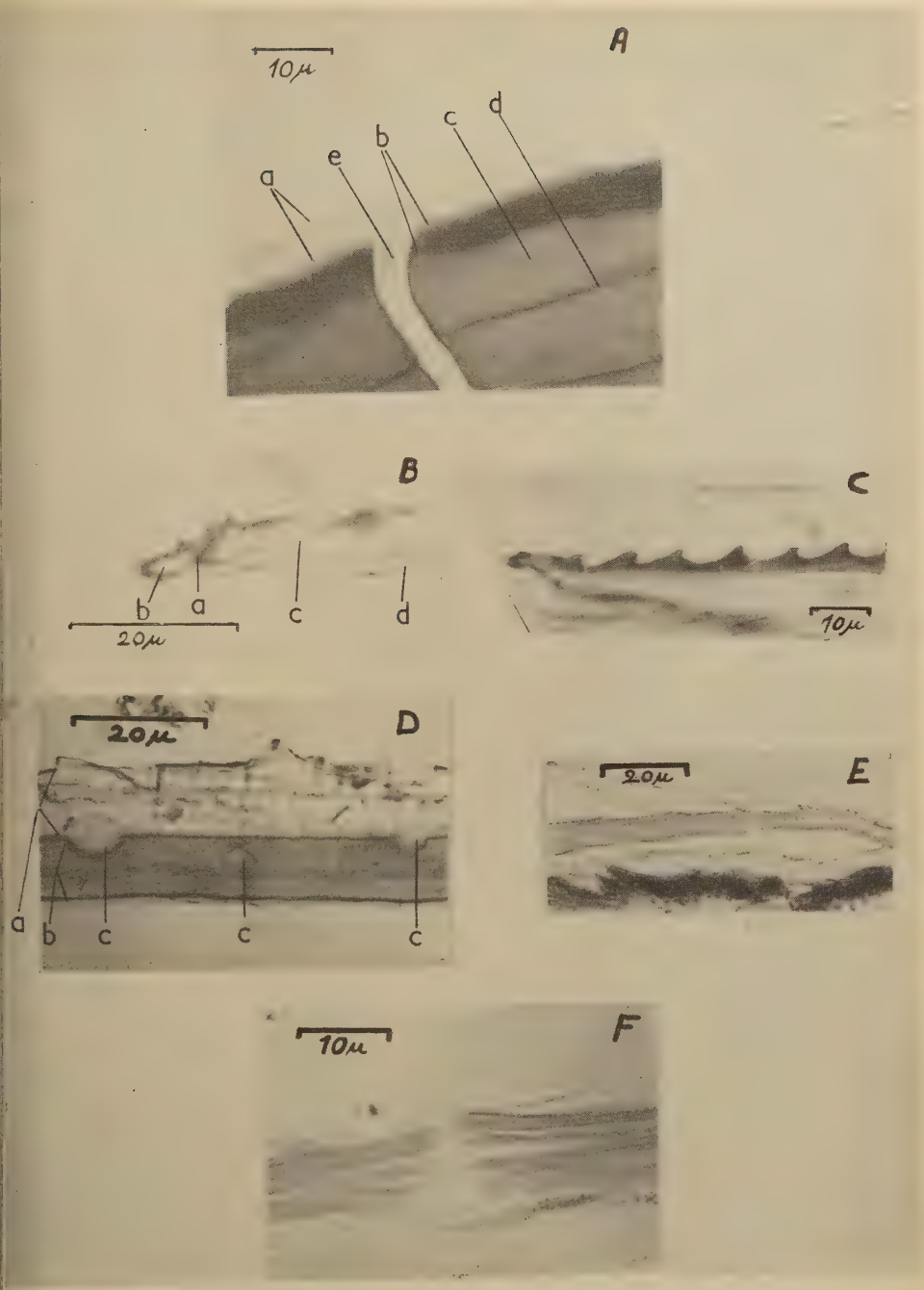
B, a section (8 μ) of the edge of a roach-scale. Toluidine blue. There is strong metachromasy in the zone of calcification (*a*) under the outermost stria, penetrating into the uncoloured osseoid (*b*). Neither the bony layer (*c*) nor the fibrillary plate (*d*) is stained.

C, a section of a scale, decalcified and stained with toluidine blue. The whole bony layer shows metachromasy.

D, the fibrillary layer in section. Toluidine blue. The upper calcified part (*a*) is practically uncoloured, the remaining uncalcified part (*b*) shows a heavy orthochromasy. The boundary between the two parts shows two Mandl-bodies (*c*); another is being formed in the uncalcified part.

E, Hotchkiss's PAS-reaction applied to a section, showing equal intensity of colour in the bony layer and in the fibrillary plate.

F, a section of the fibrillary plate, with a radius in the middle. Hotchkiss's PAS-reaction. The radius takes no stain, in contrast to the surrounding lamellae.


A
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The zone of active calcification in the bony layer is characterized by a well-marked increase in the metachromasy (fig. 1, B), the PAS-reaction, and in the reactions for bone salts.

Heller-Steinberg (1951) found an increase in the two last-mentioned reactions at the transition between calcified and uncalcified bone tissue. This was interpreted as an increase of the reactive groups. Rubin and Howard (1950) also found an enhancement of the PAS-reaction and of metachromasy in the same place, and thought this to depend on a change of state or concentration in the calcification.

TABLE 1

Reactions on Mucopolysaccharides and state of calcification of the roach-scale

The bony layer	*Ortho-chromasy*	*Meta-chromasy*	*PAS-reaction*	*Reactions for bone salts*
Normal scales				
Osseoid	○	○	○	○
Zone of calcification	○	+ + +	+ + +	+ + +
The calcified remainder	○	○	+ + +	○–+
Regenerated scales				
Osseoid	○	+ +	+	○
Zone of calcification	○	+ + +	+ +	+ + +
The calcified remainder	○–+	○	+ +	+ +
The fibrillary plate				
I. Normal scales:				
Uncalcified part	+ + +	○	+ + +	○
Calcified part	○	○	+ + +	+ +
II. Regenerated scales	○	○	○	○

+++ strong reaction; ++ moderate reaction; + feeble reaction; ○ no reaction.

The methods of Heller-Steinberg and of Stoeltzner both involve reactions with the anions of the bone salt. This may indicate that there is a relatively larger concentration of free PO_4-groups in the zone of calcification, but also the larger surface of the calcoglobules might increase the reactions. However, it has recently been shown that Ca-ions are bound to the osseoid through SO_2- and PO_4-groups (Caglioti, Ascenzi, and Scrocco, 1954). Thus it is possible that the great increase in metachromasy and in the reactions on bone salts at the zone of calcification really indicates SO_2- and PO_4-groups.

The rapidly growing regenerating scales show metachromasy to be extended to the very periphery, the rest being usually stained with weak orthochromasy, pointing to an under-saturation of calcium. This is supported by the moderate PO_4-reactions.

The absence of metachromasy and of a well-defined zone of calcification in the fibrillary plate suggests another type of calcification. The coarsely crystalline structure indicates a direct precipitation of the mineral matter from the tissue fluids. The calcification does not follow the limits of the lamellae, but proceeds faster in the peripheral than in the central parts of the scale. The

uneven boundary between calcified and uncalcified tissue thus runs at an angle across the lamellae. The age of the lamellae is of no importance to the calcification, which is governed by some unknown factor.

Acid groups of the collagen might cause orthochromatic staining. The disappearance of this staining at the calcification implies a removal or a neutralization of these acid groups before the bone salts can be precipitated. Another necessary factor is the polysaccharide, which gives a strongly positive PAS-reaction. Under the radii, where the PAS-reaction is negative no calcification occurs. However, this polysaccharide does not seem to be directly connected with the calcification, as the PAS-reaction is of the same intensity in both calcified and uncalcified lamellae.

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Mitotic Activity during the Early Differentiation of the Scleral Bones in the Chick

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Summary

The dispositions of mitoses in the mesenchyme beneath the conjunctiva of parts of a 7-, 8-, 9-, and 10-day embryo have been ascertained. For the purpose of analysis, the number of mitoses, cell population density, and mitotic index have been found in small segments of annuli which include parts of the rings of bone primordia, and the mesenchyme between them. Twenty-three primordia were studied.

It is found that the cell population density increases from about $4–14 \times 10^5$ per cubic mm within the primordia, but only from about $4–9 \times 10^5$ in the mesenchyme between them.

Mitotic activity increases sharply within young primordia from about 12–27 mitoses per 1,000 cells, and thereafter decreases. It is found that this mitotic activity probably occurs in three waves of decreasing intensity. Between the primordia mitotic activity is at a markedly lower level and probably follows a simpler pattern.

The cells involved in this mitotic activity in the primordia are particularly those just beneath the conjunctival papilla, and the number of mitoses decreases the greater the distance from the conjunctiva. In the 8- and 9-day embryos, the number of mitoses is inversely proportional to the distance.

Calculation indicates that in each of the three waves of cell division, the cells that divide do so only once; and that this mitotic activity is adequate to account for the increase in cell population density which is found.

The cause of the increase in the number of cell divisions in the primordia is not clear, but the result is probably the production of blastemas, the cells of which become the osteoblasts of the scleral bones.

Introduction

IT is well known that the eyeball of birds contains a bony ring made of a number of separate elements which lie close together. Nelson (1942) found that in the adult fowl 13–16 scleral bones occurred in each eye, 14 being the most usual number. The early development of these scleral bones in the chick has been studied by Nussbaum (1901), Dabelow (1927), and more recently by Murray (1943). The authors of these papers studied particularly the adjacent conjunctival papilla, whose growth precedes the appearance of the bones. Murray also dealt in some detail with the fate of the mesenchyme condensations which appear beneath the papillae.

The formation of a mesenchyme cell condensation, before the formation of a membrane bone, has also been described by Jacobson and Fell (1941) in the development of the avian mandible, and by Pehrson (1922, 1940, 1944*a*, 1944*b*, 1947), and Devillers (1947) in the bones which grow in close physical relationship with the neuromast organs in various fishes.

In this paper the time and place of mitoses in the bone primordia has been analysed. It is shown that the centre of mitotic activity is mainly near to the

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conjunctival papillae, and that the mesenchyme condensations are probably produced as a result of three waves of cell division.

Terminology

Primordium. The primordium of a scleral bone consists of those cells which are destined to become the osteoblasts and osteocytes of the scleral bone. It is, therefore, the group of cells lying between the conjunctival papilla and the fibrous sclera (the latter is the fibrous rim around the anterior border of the sclerotic). It is not possible to define the precise boundaries of a primordium at the early stages studied in this investigation; this difficulty has been met in a way described on p. 339.

The numbering of the primordia. The primordia have been numbered for reference (see fig. 2, p. 336). These numbers have no relation to the order in which the primordia appear in the eye; they are numbered in this way for convenience only.

Orientation. The terms 'top' and 'bottom', and 'upwards' and 'downwards', &c., are used in this paper. The top of the fibrous sclera (see fig. 1) refers to the boundary nearer to the conjunctiva, and the bottom, to the boundary away from the conjunctiva; upwards means towards the conjunctiva, and downwards away from it. 'Inwards' indicates towards the cornea, and 'outwards' away from it. The 'front of the eye' refers to that part of the eyeball which is visible externally; that is, the part covered by the conjunctiva.

Statistical abbreviations. The following have been used in recording the results of statistical tests. t, F, and χ have the usual meanings. 'D.F.' signifies the number of degrees of freedom; in variance ratio (F) tests, the number of degrees of freedom in the greater estimate (a) and in the smaller estimate (b) of the variance are written: D.F. $= a/b$. P is the approximate probability of equalling or exceeding the value of the t, F, or χ^2 obtained.

Materials

Twenty-three known primordia from 4 eyes were used in the investigation as follows: one 7-day eye, 6 primordia, plus 3 or 4 'suspected' primordia; one 8-day eye, 5 primordia; one 9-day eye, 6 primordia; and one 10-day eye, 6 primordia.

Method

Histological technique. The fronts of the eyeballs of the 7- and 8-day embryos were fixed in Zenker, and those of the 9- and 10-day embryos in Cleland's modification of Bouin's fluid. The material was dehydrated in dioxane and embedded in paraffin wax in the usual way. The sections were cut in a plane perpendicular to the front of the eye. The same microtome and knife were used for sectioning all the material at an indicated thickness of 10μ. The sections were subjected to the Feulgen technique, and counterstained in light green.

Recording of mitoses. Fig. 1 is a diagram of a section (from the 8-day eye) illustrating a scleral bone primordium, its adjacent conjunctival papilla, and other nearby structures in the eye. Outline drawings, such as this, were made of every section, and on them were marked the positions of the mitoses, as seen under the microscope. Records were made of the dividing cells found in the tissues bounded by the base of the eyelid (or nictitating membrane), the boundary of the anterior chamber of the eye, the conjunctiva, and the base of

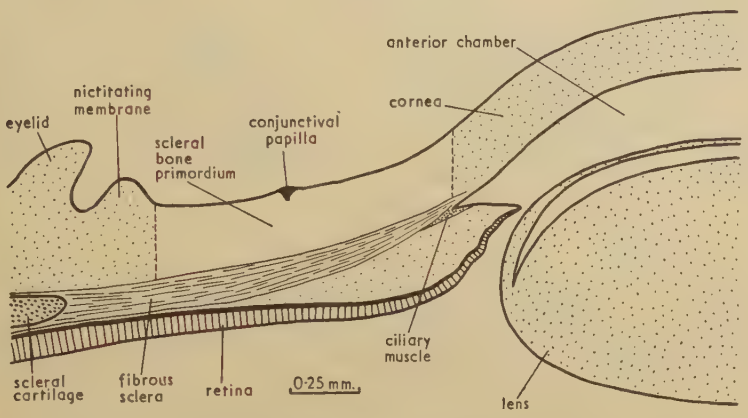

Fig. 1. A scleral bone primordium and the adjacent structures. The diagram, drawn from a section of an 8-day eye, shows a scleral bone primordium, the adjacent conjunctival papilla, and other nearby structures in the eye. The unstippled region beneath the conjunctiva is the part of the eye in which the positions of mitoses were recorded and analysed.

the fibrous sclera (unstippled region of fig. 1). A total of 631 sections in the four eyes were dealt with in this way.

Reconstructions. Those mitoses occurring in the fibrous sclera and in the lip of the scleral cartilage were obviously concerned in the development of these structures, and are, therefore, omitted from further consideration here.

Reconstructions of the data plotted in the region of mesenchyme cells between the conjunctiva and the sclera have been made in two ways in order to obtain a three-dimensional picture of the distribution of the mitoses. These are termed the 'plan' diagrams, and the 'elevation' diagrams.

(*a*) *Plan diagrams.* The plan views give the dispositions of the mitoses as if one were looking directly at the front of the eye.

On making these plan reconstructions it was found that they were distorted; the thickness of the sections was greater than the 10μ indicated by the microtome. The actual thickness of the sections was estimated by means of the fine adjustment micrometer of the microscope, which gave a mean value of 12·7μ (standard error of mean = $\pm$0·28; 36 observations). The making of reconstructions is greatly facilitated if the relative magnitudes of the two dimensions conveniently fit squared paper. Thus, 12·5μ was taken as the section thickness; it is well within the range that could be expected from these

observations (12·12–13·26, at the 0·05 level of significance), and also, the reconstructions were approximately normal in shape.

The plan views are shown in fig. 2.

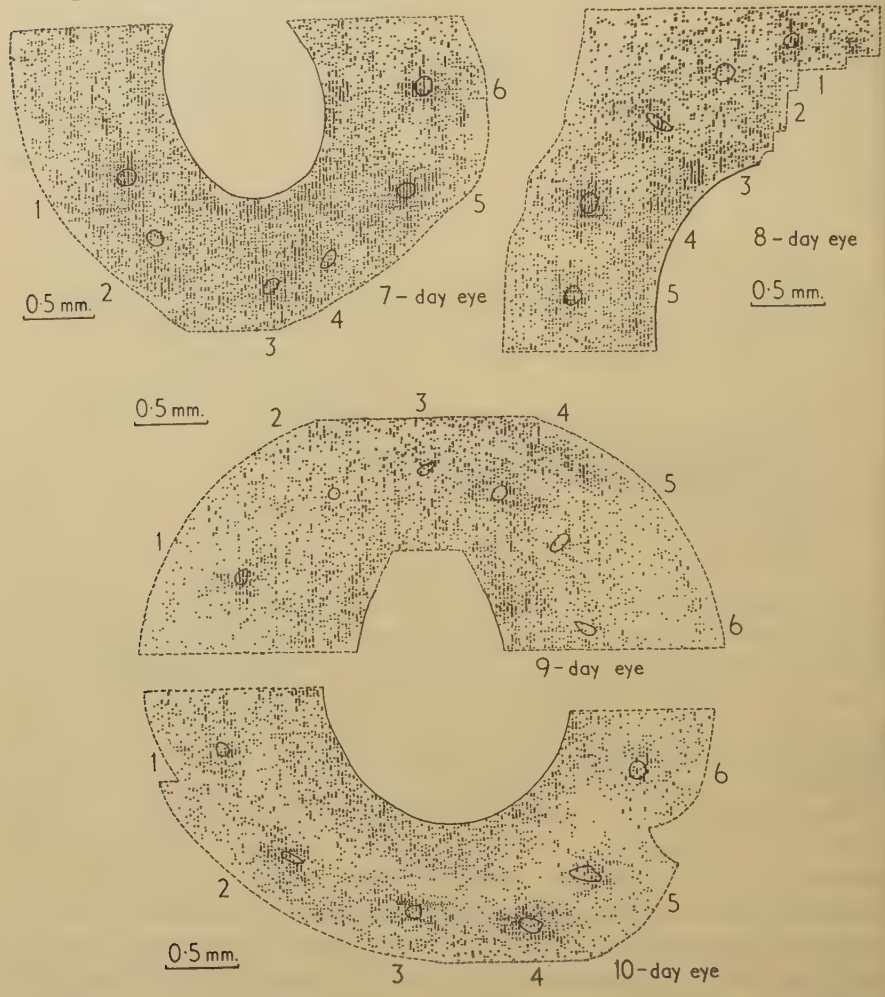

FIG. 2. Plan reconstructions of the mitoses in the mesenchyme. Each reconstruction displays the positions of mitoses as if one were looking at the front of the eye. The inner full line marks the boundary of the anterior chamber of the eye, and the outer broken line shows where the eyelids (or nictitating membrane) are attached. The numbers refer to the scleral bone primordia, the positions of which are indicated by the outlines of the conjunctival papillae. Each dot represents one dividing cell.

Note that in many primordia there are distinctive groups of mitoses.

(*b*) *Elevation diagrams.* These diagrams show the distribution of the mitoses in the mesenchyme from the conjunctiva to the fibrous sclera.

Squares (arranged circumferentially) were drawn around each papilla on the plan diagrams, as shown in fig. 3. All the mitoses within the block of tissue

outlined by such a square are included in an elevation diagram; so that one diagram reconstructs the arrangement of the dividing cells throughout one primordium.

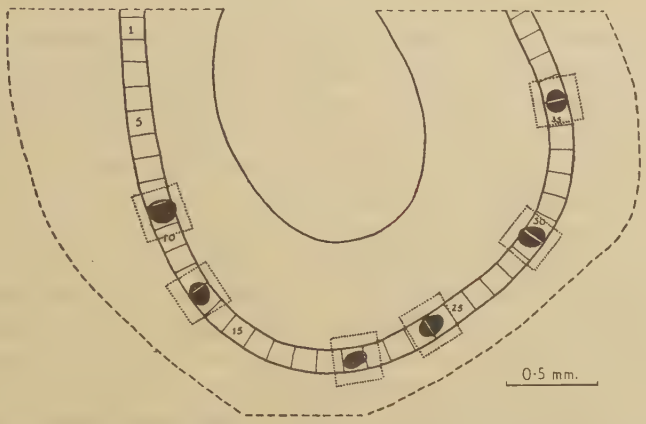

FIG. 3. Parts of the eye used in the analysis of cell population density and of mitotic activity. This diagram (of the 7-day eye) shows an annulus drawn through the ring of scleral bone primordia. In each segment of the annuli the cell population density, number of mitoses, and mitotic index were determined. The dotted squares around the scleral bone primordia (conjunctival papillae black) outline the pieces of tissue, the mitoses in which are recorded in the elevation reconstructions.

RESULTS

Plan distribution of the mitoses. The plan diagrams show that, in many cases, a greater concentration of mitoses is found near to a conjunctival papilla than in other regions. Only some primordia show this in the 7- and 9-day eyes, but all in the 8- and 10-day eyes.

In the 7-day eye it will be seen that, in addition to concentrations of mitoses near to some of the papillae, there appear to be others in places where there are no papillae. These occur between primordia Nos. 4 and 5, and possibly between Nos. 1 and 2, and before No. 1. The conjunctival papillae do not all arise simultaneously, nor are they all to be found at the same stage of development in an eye. In the 7-day eye studied, only 9 of the 14 or so papillae can be detected, the others not having begun to differentiate. In the part of the eye investigated, 9 or 10 primordia would be expected. This suggests that proliferation of the mesenchyme cells of a primordium may precede differentiation of a papilla. In the case of No. 2 of this eye, the papilla has begun to differentiate, but no cluster of mitoses is found. Thus mesenchyme proliferation may begin either before or after the papilla first appears.

In the other three eyes, no groups of mitoses are found other than near to a papilla. A greater concentration of mitoses can be seen near to the line indicating the boundary of the anterior chamber of the eye; this may be connected with the development of the cornea (as the nearest developing structure), but this apparent correlation has not been investigated.

Cell population density and mitotic index. Murray (1943) has indicated that the mesenchyme cells increase in population density within a bone primordium to form a condensation; thus one might expect to find a greater number of mitoses within them without this necessarily being significant. It is thus desirable, not only to count the dividing cells, but also to measure the cell population density. These two parameters can then be combined to calculate another measure of mitotic activity, in which the effect of changing cell population density is eliminated. For this third measure the number of dividing cells found among a standard number of cells (1,000) has been used. This is the mitotic index, which was first used as a measure of rate of growth by Minot (1908), and has since been employed by numerous workers.

These three parameters (cell population density, number of mitoses, and mitotic index) were estimated for certain regions of the mesenchyme beneath the conjunctiva. On the plan diagram for each eye was drawn an annulus of known width (0·125 mm) as shown in fig. 3 (this is for the 7-day eye). Each annulus was then divided into segments of equal size, which, for all practical purposes, were squares of side 0·125 mm. For each of the blocks of mesenchymatous tissue outlined by a segment of the annulus, the cell population density, number of mitoses, and mitotic index were determined.

The cell density was measured by counting the number of nuclei seen within a known area of an eyepiece graticule. The average density over the whole segment was estimated by taking the mean of readings in five different places. Such a count gives an over-estimate of the density, since a nucleus bisected in sectioning will appear in two sections. By measuring nuclear diameters, the population density measurements have been corrected for this error (Abercrombie, 1946).

Another possible error in the estimation of the cell population densities arose from the discovery that the embryos from which the mitotic data was extracted had, unfortunately, been fixed in two different fixatives; it is unlikely that both fixatives will shrink the tissues to exactly the same extent. Some data have been published (Patten and Philpott, 1921) on the shrinkage due to fixing in Zenker's fluid, but none for Cleland's modification of Bouin's fluid. Ross (1953) has also studied shrinkage due to fixation, but his results also give no help in this case. The relative shrinkage caused by these two fixatives on this material was estimated by making measurements of the fronts of the eyes of 12 embryos before and after fixation. Three were fixed after 7, 8, 9, and 10 days of incubation respectively; one eye from each embryo being fixed in Zenker, and the other in Cleland. If the original size of the material is 1, after fixation in Zenker the average size was found to be 0·82 (standard deviation = 0·04), and after fixation in Cleland 0·89 (S.D. = 0·04). The difference between these means is statistically significant ($t = 2{\cdot}9$, D.F. = 22, and $P = 0{\cdot}01$). If the size of the material after Cleland fixation is 1, then the average relative size of the material after Zenker fixation becomes 0·93, with a range of 0·80–1·06 (at the 0·05 level of significance).

Thus, the differential effect of the two fixatives could be to some extent

removed by modifying the cell population densities of the material fixed in Zenker by this factor of 0·93. The range of possible values suggested that a better estimate for the particular embryos used should, if possible, be made, and this was done in the following way. The cell population density has been plotted against the mitotic index for each segment of an annulus (see fig. 4, B, p. 345) included within a primordium. It was found that the average curves for each eye had similar shapes. The 'humps' on the curve for the 7-day eye were similar in position to those of the 8-day eye; and those for the 9-day eye similar to those of the 10-day eye. But the average curves for the two pairs differed in that the curve for the 7- and 8-day eyes was to the right of the curve for the 9- and 10-day eyes; that is, the cell population densities were higher for the younger pair. This difference is almost certainly due to the different amount of shrinkage in the two fixatives. Reduction by 0·85 of the cell densities estimated from the material fixed in Zenker brought the two sets of curves together.

Comparison of this factor (0·85) with the average relative shrinkage found above (0·93) shows that this value could be obtained in about 1 in 4 embryos ($t = 1{\cdot}25$, D.F. = 11, and $P = 0{\cdot}24$). The cell population densities of the material fixed in Zenker were therefore multiplied by this factor of 0·85 so as to bring them into line with those of the material fixed in Cleland.

The variability in shrinkage of the same types of tissue in one fixative makes it clear that, had all eyes been treated with the same fixative, the cell population density measurements would be likely to exhibit a similar variability to that shown by these corrected measurements. This is only one source of variability among many: two others (section thickness, and bisection of cells during sectioning) have been reduced. The remaining variability determines the amount and precision of information which can be revealed by statistical analysis.

The absolute values of cell population density quoted here are probably a little higher than in the living tissues, owing to shrinkage during fixation. The variability in shrinkage makes a more accurate estimation difficult and unprofitable. While pointing out the inaccuracy of these densities, it should be noted that the analyses recorded in this paper involve comparisons between embryos, and between scleral bone primordia; in this respect, the inaccuracy is unimportant.

The cell population density provides a means of estimating the number of cells within a segment of an annulus, since the volume of the piece of tissue can be easily calculated. The number of mitoses occurring in the segment can be counted from the plan diagrams, and thus the mitotic index determined.

The blocks of tissue outlined by the annuli have been separated into those occurring within the primordia, and those between the primordia; but, as previously stated (p. 334), this segregation cannot be accomplished with certainty, especially in the earlier stages. Each primordium is located as being beneath a conjunctival papilla. The extent of the condensation of mesenchyme cells and the region of apparently increased mitotic activity were then used to

allocate segments of an annulus to a primordium. This was usually two segments, but occasionally one or three were allocated. Such a procedure, however, partly anticipates the results of the analyses which follow. But it should be noted that every segment is included in either the 'between primordia' or the 'within primordia' categories, and that the results are based on comparisons of the readings in these two regions. In addition, I have repeated the analyses with alternative estimates of the extent of those primordia whose limits were more difficult to judge; the conclusions indicated by these analyses proved to be the same as those recorded here.

Individual primordia. Each primordium was first tested to judge whether there was justification for including it in the 'within primordia' category, this being especially necessary in the cases of the 'suspected' primordia in the 7-day eye which had no adjacent conjunctival papilla. The mean of the cell population density, numbers of mitoses, and mitotic index of a primordium, over the segments of the annulus allocated, were compared individually with the mean of the 'between primordia' data of the eye.

All the 'known' primordia had a significantly different mean of one or more of these parameters. Of the 'suspected' primordia in the 7-day eye, only that between Nos. 4 and 5 could be included as a primordium; this was because of its relatively high number of mitoses and mitotic index. In fact, the mitotic index within this suspected primordium was higher than in any other primordium (34·5), perhaps indicating that the onset of the higher mitotic activity shown to take place (see p. 343) is sudden and intense. In the other two suspected cases, the differences are small enough to be either just random variations, or else they are very young primordia in which the differences are not sufficiently large to distinguish them from random fluctuations.

Analysis of changes in cell population density. (*a*) *Between primordia.* A linear regression line has been fitted to the cell population densities, with the age of the embryo as the independent variable. The slope of this regression line departs significantly from zero ($t = 6{\cdot}72$, D.F. $= 74$, and $P < 0{\cdot}001$); an increase in the cell population density takes place as the embryo gets older.

Further analysis shows that the cell population density only increases from 8–9, and from 9–10 days of incubation, as is shown in table 1.

(*b*) *Within primordia.* The straight line regression fitted to the 'within primordia' data, the age of the embryo being used as the independent variate, again shows a significant departure from a horizontal line; the cell density increasing with time ($t = 5{\cdot}79$, D.F. $= 55$, and $P < 0{\cdot}001$).

Further analysis shows that the increase which takes place is not regular, and table 2 records comparisons of the mean cell population density between successive days. Included in this table is the mean cell population density between the primordia of the 7-day eye, this being taken as the cell population density immediately before the primordia become established.

There is thus an initial rise in the cell population density, and a further rise between 8 and 9 days of incubation.

(*c*) '*Between primordia*' *data compared with* '*within primordia*' *data.* Com-

parison of the slopes of the two regression lines discussed in (*a*) and (*b*) above, shows that they are probably different ($t = 1{\cdot}96$, D.F. = 129, and $P \simeq 0{\cdot}05$). The mean cell population density within the primordia ($9{\cdot}02 \times 10^5$ cells per

TABLE 1

Cell population density changes. 'Between primordia' data

Age of eye (days)	*Cell population density*			*Comparison between successive days*		
	Mean $\times 10^5$ *per c.mm*	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7	5·6	±0·25	23			
				1·28	35	0·3>*P*>0·2
8	5·1	±0·31	14			
				4·30	29	<0·001
9	6·8	±0·24	17			
				2·95	37	0·01>*P*>0·001
10	8·0	±0·32	22			

cubic mm) differs significantly from that between the primordia ($6{\cdot}47 \times 10^5$) ($t = 6{\cdot}88$, D.F. = 131, and $P < 0{\cdot}001$). Thus, not only is the cell population density higher within the primordia, but the rate of increase is also probably greater. There may also be a significant rise in density in the very young primordia.

TABLE 2

Cell population density changes. 'Within primordia' data

Age of eye (days)	*Cell population density*			*Comparison between successive days*		
	Mean $\times 10^5$ *per c.mm*	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7*	5·6	±0·25	23			
				3·45	37	0·01>*P*>0·001
7	7·3	±0·45	16			
				0·04	26	1·00>*P*>0·9
8	7·1	±0·32	12			
				4·75	24	<0·001
9	10·8	±0·66	14			
				0·01	27	1·0>*P*>0·9
10	10·7	±0·52	15			

* 'Between primordia' data.

Analysis of the changes in the numbers of mitoses. (*a*) *Between primordia.* A linear regression line of the number of mitoses in the segments of the annuli, with the age of the embryo as the independent variable, departs significantly from zero ($t = 3{\cdot}48$, D.F. = 74, and $P < 0{\cdot}001$); the number of mitoses decreases with increasing age.

Day-to-day analyses show that the decrease is not entirely uniform, there

being a bigger, significant change between 8 and 9 days of incubation. The results of these comparisons are shown in table 3.

(*b*) *Within primordia.* The linear regression line (with age the independent variable) again differs significantly from zero ($t = 4{\cdot}50$, D.F. $= 55$, and $P < 0{\cdot}001$).

TABLE 3

Changes in the number of mitoses in the blocks of the annuli. 'Between primordia' data

	Number of mitoses (in blocks of annuli)			*Comparison between successive days*		
Age of eye (days)	*Mean*	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7	14·9	±1·07	23			
				1·28	35	0·2>*P*>0·1
8	13·1	±0·51	14			
				2·60	29	0·02>*P*>0·01
9	9·0	±1·36	17			
				0·87	37	0·4>*P*>0·3
10	10·6	±1·15	22			

Day-to-day analyses are recorded in table 4, which also includes the 'between primordia' data of the 7-day eye to indicate the initial mean number of mitoses.

TABLE 4

Changes in the number of mitoses in the blocks of the annuli. 'Within primordia' data

	Number of mitoses (in blocks of annuli)			*Comparison between successive days*		
Age of eye (days)	*Mean*	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7*	14·9	±1·07	23			
				5·89	37	<0·001
7	30·1	±2·22	16			
				1·20	26	0·3>*P*>0·2
8	26·2	±2·28	12			
				3·45	24	0·01>*P*>0·001
9	16·6	±1·67	14			
				5·69	26	<0·001
10	19·6	±1·75	15			

* 'Between primordia' data.

In table 4 it is seen that the number of mitoses increases markedly in the youngest primordia, and after remaining at a high level, it decreases sharply at 9 days of incubation. This is followed by a second significant rise in 10-day primordia.

(*c*) *'Between primordia' data compared with 'within primordia' data.* The rate

of decrease in the number of mitoses within the primordia is probably greater than between them ($t = 2{\cdot}35$, D.F. = 129, and $P \simeq 0{\cdot}02$). The average number of mitoses is greater within the primordia (23·2, as compared with 12·0 between the primordia) ($t = 8{\cdot}84$, D.F. = 131, and $P < 0{\cdot}001$). The day-to-day analyses reveal that between primordia, the only significant change is a decrease from 8 to 9 days, a similar decrease also taking place within the primordia. The latter data shows a big initial increase in the number of mitoses, and also an increase in 10-day primordia.

Analysis of the changes in mitotic index. The mitotic index, as stated previously, is a measure of mitotic activity in which the effect of changing cell population density is eliminated.

TABLE 5

Changes in the mitotic index. 'Between primordia' data

	Mitotic index			*Comparison between successive days*		
Age of eye (days)	*Mean*	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7	12·6	±0·88	23			
				1·63	35	$0{\cdot}2 > P > 0{\cdot}1$
8	10·3	±1·12	14			
				1·55	29	$0{\cdot}2 > P > 0{\cdot}1$
9	7·6	±1·28	17			
				0·49	37	$0{\cdot}7 > P > 0{\cdot}6$
10	6·9	±0·71	22			

(*a*) *Between primordia.* The regression line against the age of the embryo is highly significant ($t = 4{\cdot}86$, D.F. = 74, and $P < 0{\cdot}001$), and shows a decrease of mitotic index with increasing age. Day-to-day analyses show that the decrease is gradual, there being no significant change between successive days.

(*b*) *Within primordia.* The regression line against time departs significantly from zero ($t = 4{\cdot}24$, D.F. = 55, and $P < 0{\cdot}001$), showing a decrease in mitotic index with increasing age of embryo. In the day-to-day analyses recorded in table 6, the 'between primordia' data for the 7-day eye is again included as an indication of the initial mitotic index within primordia.

There is thus a sharp initial increase in mitotic index, which is probably followed by a decrease between 7 and 8 days of incubation. The decrease from 8 to 9 days is of doubtful significance.

(*c*) *'Between primordia' data compared with 'within primordia' data.* The slopes of the two regression lines do not differ significantly ($t = 0{\cdot}96$, D.F. = 129, and $0{\cdot}4 > P > 0{\cdot}3$), but the mean mitotic index within primordia (13·2) is significantly higher than the mean between primordia (9·4) ($t = 3{\cdot}85$, D.F. = 131, and $P < 0{\cdot}001$). Thus, although mitotic activity falls at the same rate in both regions of the eye, it is notably higher within the primordia. The day-to-day analyses reveal no marked changes, apart from the indications of a sharp rise in the youngest primordia.

The relationship between mitotic index and cell population density. In the mesenchyme of the bone primordia, and also between them, it has been shown that the cell population density increases during the period under investigation; within the primordia, however, this increase is very much greater. During this time mitotic activity, as measured by the mitotic index, slowly decreases between the primordia; while within them it appears to fluctuate in a different way, and is at a markedly higher level. It is of interest to inquire if, and in what way, the observed mitotic activity is related to the rise in cell population density.

TABLE 6

Changes in the mitotic index. 'Within primordia' data

Age of eye (days)	*Mitotic index*			*Comparison between successive days*		
	Mean	*Standard error of mean*	*Number of observations*	*t*	*D.F.*	*P*
7*	12·6	±0·88	23			
				3·75	37	<0·001
7	19·0	±1·58	16			
				2·50	26	0·05>*P*>0·01
8	13·4	±1·43	12			
				1·74	24	0·1>*P*>0·05
9	9·8	±1·44	14			
				0·01	27	1·0>*P*>0·9
10	10·0	±0·88	15			

* 'Between primordia' data.

(*a*) *Between primordia.* A regression line fitted to the mitotic index (dependent variable) and cell population density (independent variable) is significant ($t = 2{\cdot}98$, D.F. $= 74$, and $0{\cdot}01 > P > 0{\cdot}001$), showing a progressive decrease in mitotic index as the cell population density increases. In fig. 4, A the trend of the relationship is shown by the polygon joining means of mitotic index over small ranges of cell population density. It shows that the mitotic index is relatively high (about 12–15) between cell densities of about 4·0 and $5{\cdot}5 \times 10^5$ per cubic mm, and is considerably less (about 8) at the higher cell population densities.

(*b*) *Within primordia.* A similar regression line fitted to the cell population density and mitotic index data is also highly significant ($t = 6{\cdot}39$, D.F. $= 55$, and $P < 0{\cdot}001$), again showing a decrease in mitotic index with increasing cell population density (fig. 4, B). The trend line in this case shows different features from that of the 'between primordia' data: as the cell population density increases, the mitotic index rises to a peak, falls, and rises again to a rather smaller but still pronounced maximum, and this may be followed again by a third rather flat 'hump'. In order to determine whether these variations are real, or whether they are just random variations, the data were subjected to the following treatment.

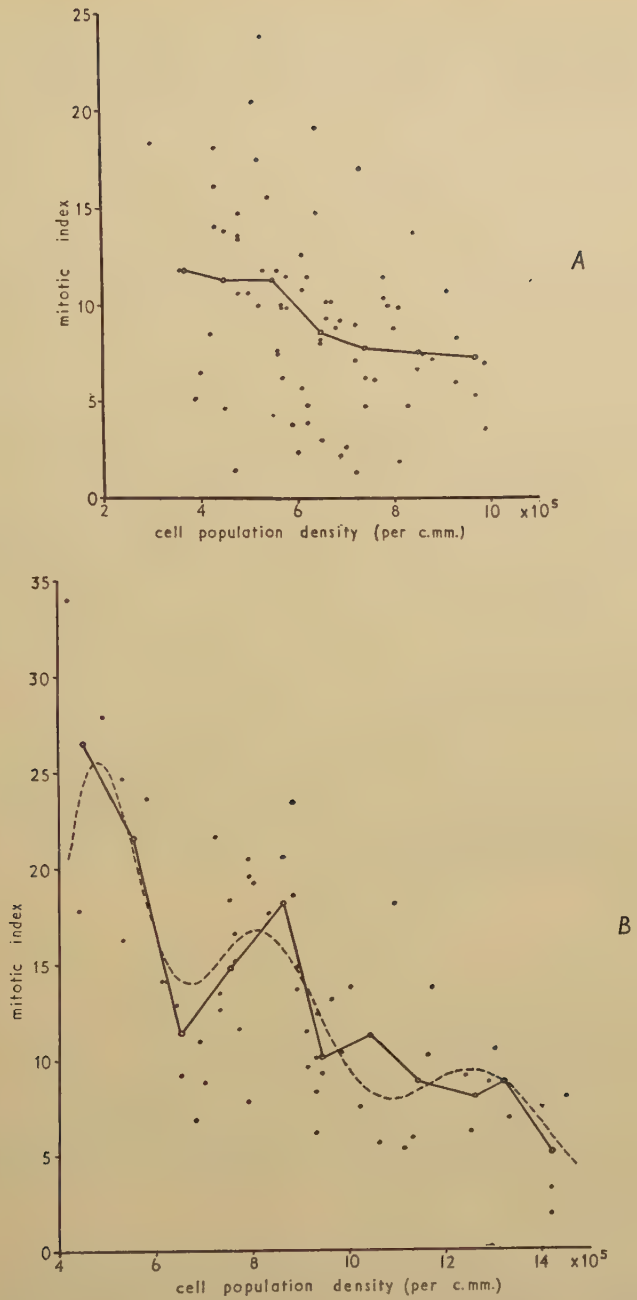

FIG. 4. The relationship between cell population density and mitotic index. Each point represents the mitotic index and cell population density for a segment of an annulus. The full line connects the mean mitotic indices over the ranges of cell population densities 3·0–3·9, 4·0–4·9, &c. The broken line in B is a damped cosine curve fitted to the 'within primordia' data. A, 'between primordia' data. B, 'within primordia' data. Note the larger range of cell population density and of mitotic index within the primordia, and the markedly different relationship of these two parameters in the two sets of data.

A theoretical type of curve was required in which, over this range of cell population density, the ordinates (mitotic index) would rise and fall in a fashion similar to that indicated by the observations. The type of curve selected was

$$y = -\frac{\cos x}{x},$$

and the data was used to find the best fit of such a curve. The general equation of this type of curve is

$$y = -a\frac{\cos \lambda x}{x} + b$$

where a (the amplitude), b (correction for the origin), and λ (the wavelength) are constants to be estimated from the data.

The trend line indicated that three wavelengths were needed to correspond with the 'humps' on the curve, but that the wavelengths became progressively longer. By trial and error, it was found that they became approximately equal if the cell population density was transformed by taking its cube root; one wavelength corresponded to an interval of 0·32 on the transformed scale ($= \lambda$). The origin of the curve was taken at a cell population density of $3{\cdot}7 \times 10^5$ per cubic mm ($\sqrt[3]{3{\cdot}7} = 1{\cdot}55 = b$). The cube roots of the densities were then converted to angular measure.

A straight line regression was fitted to this transformed data (it was significant; $t = 6{\cdot}64$, D.F. $= 55$, and $P < 0{\cdot}001$). The cosine curve was then fitted to the deviations of the observations from this fitted straight line. The origin and wavelength having been estimated empirically, the curve reduces to a straight line

$$y = -aX \quad \text{where} \cdot \frac{\cos \lambda x}{x} = X$$

so that the amplitude may be estimated by the least squares method. The fitted curve is shown, in the original units, in fig. 4, B.

Analysis of variance shows that the variance of the observations around the cosine curve is significantly less than that around the linear regression line ($t = 3{\cdot}09$, D.F. $= 54$, and $0{\cdot}01 > P > 0{\cdot}001$), showing that the cosine curve is the better representation of the relationship between mitotic index and cell population density.

Thus, while the cell population density rises from about $4\text{–}14 \times 10^5$ per cubic mm, three waves of mitotic activity of successively decreasing intensity probably occur.

In this analysis the independent variable, against which the mitotic index has been analysed, is the cell population density, not time. The cell population density was shown earlier to increase with the age of the embryo, but it was also shown that the density increased mainly in only two of the four daily intervals. It is therefore obvious that the pattern of the changes in mitotic

index will not be the same against a time scale. The change of pattern, however, cannot eliminate the 'humps' and 'troughs' in this curve; it will either extend them or contract them.

The age of the embryo was the most accurate time-scale available, but the units are too large to demonstrate the smaller changes in mitotic index which are seen to occur from the analysis against cell population density. The known development stages of a nearby structure can form a time-scale of smaller unit, and the growth and recession of the conjunctival papilla has been used for this purpose. It confirmed that mitotic activity passed through three successive peaks of decreasing intensity.

The elevation distribution of the mitoses. The dispositions of the mitoses within each primordium has been plotted to show their arrangement in elevation, that is, with reference to the conjunctiva, fibrous sclera, conjunctival papilla, &c. An example from each eye is shown in fig. 5.

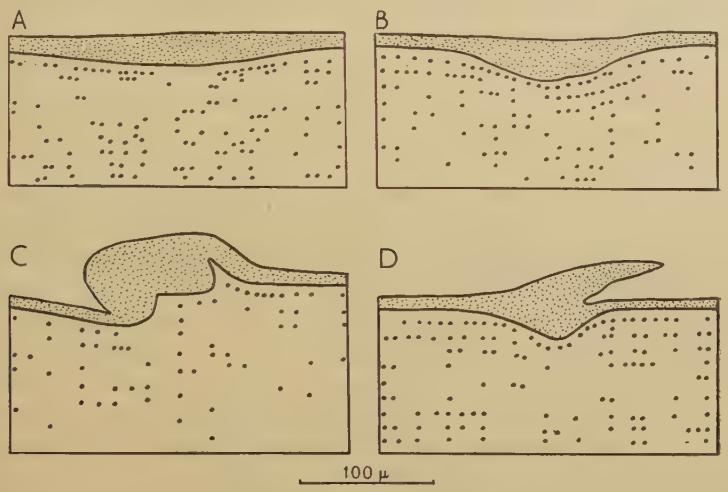

FIG. 5. Elevation reconstructions of mitoses in the primordia. The conjunctival papilla is shown at the top of each diagram (stippled), and the horizontal line at the bottom represents the mesenchyme-sclera boundary. Each dot represents one dividing cell. A, 7-day eye, No. 1; B, 8-day eye, No. 5; C, 9-day eye, No. 3; D, 10-day eye, No. 3. In one or two of the 7-day primordia (A), the mitoses are fairly evenly distributed, but in most of the others (B, C, and D) there is a preponderance of dividing cells near to the conjunctival papilla. In some 10-day primordia (D), the number of mitoses near to the mesenchyme-sclera boundary increases.

In one or possibly two primordia of the 7-day eye, the mitoses are more or less evenly distributed (fig. 5, A). In the other primordia of this eye and in those of the other three eyes, there is a marked preponderance of mitoses near the conjunctival papilla; this feature is less prominent in some 9-day, and in most 10-day primordia (figs. 5, B, C, and D). In the 10-day eye, there is, in addition, a noticeable increase in the number of mitoses near the sclera.

The elevation distribution has been analysed quantitatively. The logarithm of the number of mitoses appeared to vary as the logarithm of the distance

from the conjunctiva; that is, the number of mitoses (y) might be related to the distance (x) by a curve of the type

$$y = ax^b$$

or

$$\log y = \log a + b \log x,$$

a and b being constants calculable from the data. Curves of this type were fitted (least squares method) to the data for each primordium, to the grouped data for each eye, and to all the data; and regression and covariance analyses were carried out.

In the 7-day eye, in primordium No. 1, such a regression line is not significant ($t = 2{\cdot}13$, D.F. $= 7$, and $0{\cdot}1 > P > 0{\cdot}05$), but in all other primordia, the slopes of the lines differed significantly from zero (t varying from 3·2–8·1, D.F. $= 7$, and $P \leqslant 0{\cdot}01$). Comparison of these regression lines showed that the differences between them were probably random ones ($F = 1{\cdot}01$, D.F. $= 5/42$, and $P > 0{\cdot}2$).

In the 8-day eye the regression lines are all significant (t varying from 4·12–6·72, D.F. $= 7$, and $P \leqslant 0{\cdot}01$). Comparison of the regression lines showed once again that the differences could be random variations ($F = 2{\cdot}15$, D.F. $= 4/35$, and $0{\cdot}2 > P > 0{\cdot}05$).

The analysis of the 9-day distribution gave similar results to those of the 8-day eye. In individual primordia the regression lines were all significant (t varying from 2·53–6·14, D.F. $= 7$, and $P \leqslant 0{\cdot}04$), and the differences between the slopes not significant ($F = 1{\cdot}54$, D.F. $= 5/42$, and $P > 0{\cdot}2$).

In the 10-day eye, in only two primordia (Nos. 2 and 6) does this type of regression line have a slope differing significantly from zero (in both cases $t = 2{\cdot}89$, D.F. $= 7$, and $0{\cdot}05 > P > 0{\cdot}02$). In all other primordia such lines are not significant (t varying from 0·44–2·02, D.F. $= 7$, and $P > 0{\cdot}05$).

When all the data of an eye are grouped together, in each case the logarithmic curve is significant (t varies from 4·19–8·73, D.F. $= 43$–52, and $P < 0{\cdot}001$). The slope of the line is −0·71 in the 7-day eye, −0·98 in the 8- and −1·00 in the 9-day eyes, and −0·54 in the 10-day eye. Comparison of the slopes of these fitted lines show that there is no evidence of a difference between the 8- and 9-day eyes ($t = 0{\cdot}29$, D.F. $= 95$, $0{\cdot}9 > P > 0{\cdot}8$), but there is probably a difference between the slopes of the 7-day eye and of the grouped 8- and 9-day data ($t = 1{\cdot}99$, D.F. $= 149$, $0{\cdot}05 > P > 0{\cdot}02$), and almost certainly a difference when the 10-day data is compared with the grouped 8- and 9-day data ($t = 2{\cdot}92$, D.F. $= 149$, $0{\cdot}01 > P > 0{\cdot}001$).

It was shown earlier (table 4) that the mean number of mitoses decreases from 7 to 8, and from 8 to 9 days, but increases from 9 to 10 days. These results, coupled with the changes in slope of the regression lines, shows that the decrease in the number of mitoses from 7 to 8 days takes place at greater distances from the conjunctiva; while from 8 to 9 days there is a general decrease at all distances. At 10 days the number of mitoses nearer to the fibrous sclera must certainly increase.

Fig. 6 illustrates the elevation distribution of mitoses in each eye in graphical form; the fitted logarithmic curves are also shown.

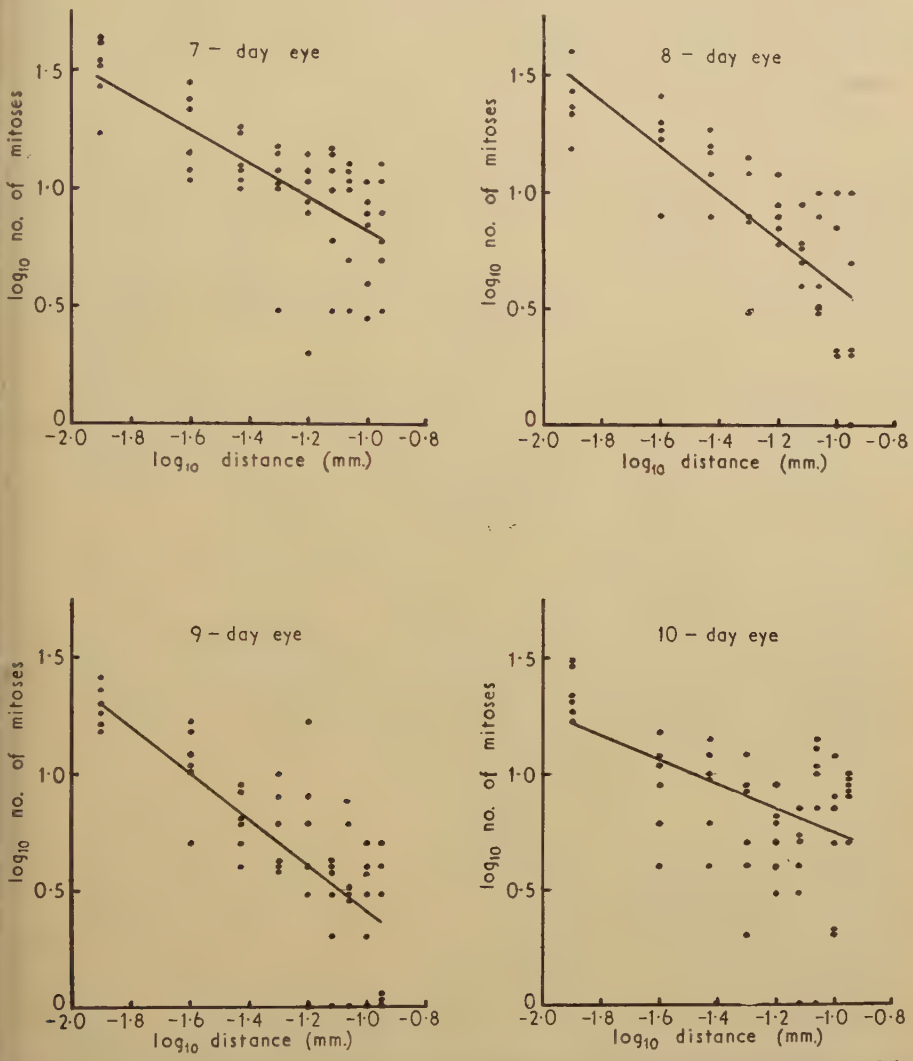

FIG. 6. Graphical illustration of the elevation distribution of the mitoses. The logarithm of the number of mitoses (ordinates) is plotted against the logarithm of the distance from the conjunctiva (abscissae) for each eye. The straight line passing through the points has been calculated from the data by the method of least squares. In the 8- and 9-day eyes the number of mitoses settles down to be inversely proportional to the distance, although the mean number of mitoses is less in the 9-day eye. The rate of decrease is less than this in the 7-day eye, and much less in the 10-day eye. In the latter case this is due to an increase in the number of mitoses at the greater distances from the conjunctiva.

Synopsis of the results of the statistical analyses. (*a*) *Cell population density.* Both within the bone primordia and in the mesenchyme between them, increases in the cell population density take place in the period under investiga-

tion; but the increase is more rapid within the primordia, and the mean density is higher. Between the primordia this increase takes place in the 8–10-day period; whereas within the primordia there appears to be a big increase in the youngest primordia, and again between 8 and 9 days of incubation (see tables 1 and 2).

(*b*) *Numbers of mitoses.* A general decrease takes place both within and between primordia; it is more rapid within the primordia. The average level of cell division is much higher within the primordia. In both sets of data, the biggest fall in number takes place from 8 to 9 days, but there may be a large initial jump in the number of mitoses within the youngest primordia (tables 3 and 4).

(*c*) *Mitotic index.* This decreases as the age of the eye increases at about the same rate in both sets of data, although the mitotic index is much higher within the primordia.

(*d*) *Relationship between cell population density and mitotic index.* Both between and within the primordia, the increase in cell population density is accompanied by a decrease in mitotic activity. Between the primordia, the mitotic index, after being relatively high at low cell densities, falls as the cell density increases further (fig. 4, A). Within the primordia, as the cell population density increases, three successive periods of greater mitotic activity of decreasing intensity probably occur (fig. 4, B).

(*e*) *Elevation distribution of the mitoses.* In most of the primordia studied there are many mitoses near to the conjunctival papilla, and fewer at greater distances from it. In one or two young primordia the mitoses are more or less evenly distributed at all distances. In the 8- and 9-day primordia the number of mitoses is probably inversely proportional to the distance. In some 10-day primordia there is an increase in the number near to the conjunctiva.

Discussion

The investigation has shown that in the bone primordia there is a rise in cell population density from about 4×10^5 to about 14×10^5 per cubic mm from the 7th to the 10th day of incubation. This rise mainly takes place in the youngest primordia, and between the 8th and 9th day of incubation.

During this time mitotic activity, initially about 12 divisions per 1,000 cells, rises suddenly in very young primordia to about 30, and thereafter decreases to 9–10 per 1,000 by the 10th day. Further analysis has shown that the changes in mitotic index are probably related to the changes in the cell population density; as the density rises from 4 to 14×10^5 cells per cubic mm, the mitotic activity probably rises and falls three times with successively less intensity.

These changes which take place within the bone primordia are of a different degree of intensity from those taking place between the primordia. They also appear to be of a different pattern, as demonstrated by the changes in mitotic index as the cell population density rises.

The use of the mitotic index for the comparison of the mitotic activity in different pieces of tissue, or in the same piece of tissue at different times, calls

for some care; a greater mitotic index does not necessarily indicate greater mitotic activity. If, in the one piece of tissue, the time for a mitosis is longer than in the other, and the 'resting' period proportionately shorter (the total time for mitosis and 'resting' period being the same for both), then a greater mitotic index will be observed. But it will be obvious that the frequency of cell division, and thus the rate of increase in the number of cells, is the same in both cases.

In the mesenchyme beneath the conjunctiva of the eye, it is seen that a higher mitotic index within the bone primordia is accompanied by a more rapid rise in cell population density, when compared with the changes taking place between the primordia. In addition, it seems unlikely that, in these cells, the average time necessary for a cell to divide is different in the two adjacent regions of the eye.

It is of interest to determine whether the mitotic activity observed could give rise to this increase in cell population density. If, among an initial number of cells x_0, $y\%$ divide, then after n divisions, the number of cells x_n is given by

$$x_n = x_0\left(1+\frac{y}{100}\right)^n.$$

The number of cells dividing in the bone primordia depends, as has been shown, on the distance from the conjunctiva. If it is assumed that, in each phase of cell division, all the cells immediately adjacent to the conjunctiva divide, then the percentage dividing at increasing distances from the conjunctiva is easily determined from the observed elevation distribution of the mitoses. After 1, 2, and 3 divisions of the dividing cells, the total number of cells is thus found to be $1{\cdot}4x_0$, $2{\cdot}0x_0$, $3{\cdot}0x_0$ respectively. The mean cell population density of two obviously very young primordia in the 7-day eye (No. 2, and the suspected one between Nos. 4 and 5) is $4{\cdot}5\times10^5$ per cubic mm; and if this is taken as the initial cell population density (x_0), then the cell population density after 1, 2, and 3 cell divisions becomes 6·3, 9·0, and $13{\cdot}5\times10^5$ per cubic mm respectively. From fig. 4, B, which relates the mitotic index variations to the variations in cell population density, it will be seen that these figures are in fair agreement with the cell population density after each of the three waves of cell division. The conclusions to be drawn from this are, first, that the mitotic activity observed could account for the increase in the cell population density; and secondly, that each cell that divides does so only once in each wave of cell division.

These results diminish the possibility that cell migration from other parts of the eye contributes to the increase in the cell population. There is no evidence that any such migration takes place, but no special inquiry has been carried out to investigate the possibility.

A relationship between the number of mitoses and the distance from the conjunctiva of the type $y = ax^b$ adequately describes the dispositions of the mitoses within the primordia, and appears to point to the conjunctival papilla

as the cause of the rise in mitotic activity. However, the fact that the papilla may appear either before or after the appearance of a larger number of mitoses, and also that the papilla grows at the same time as cell division in the mesenchyme is taking place, indicates that some third factor might stimulate both occurrences. In the 8- and 9-day eyes it is shown that the number of mitoses has settled down to be inversely proportional to the distance from the conjunctiva; and it is tempting to postulate that the cause of the mitotic activity is due to some substance diffusing from a centre at or very close to the conjunctival papilla. Such a theory infers that the intensity of cell division is directly related to the concentration of this diffusing substance; but the frequency of cell division is a shaky yardstick with which to measure this concentration. Further data are necessary to harden confidence in such a theory.

In the 10-day eye it has been shown in the analysis of the elevation distribution of mitoses that an increase in mitotic activity near to the sclera takes place, as compared with younger eyes. Murray (1943) states that from 9 to 10 days of incubation the cells of the mesenchyme condensation move from a position near to the conjunctiva towards the sclera, where they then spread outwards in a plane parallel to the conjunctiva and sclera, and just above the sclera. Thus, the mitoses seen near to the sclera probably occur among the osteogenic cells concerned in this local migration. It is at this time that the first osteoblasts differentiate from the mesenchyme (Murray), and they lay down the first fibres of the bone. The disposition of the mitoses makes it improbable that it is the osteoblasts which divide. In the plan diagram of the 10-day eye (fig. 2, especially No. 4), it is seen that mitoses are relatively rare in the centre of the group; the elevation reconstruction of a 10-day primordium (fig. 5, D) also illustrates this arrangement. The osteoblasts, as yet only few in number, are found in this central region.

The occurrence of this local cell migration, with its possibility of effecting small changes in cell population density, and the occurrence of mitoses near the sclera in the older primordia, may also be reasons for the relative vagueness of the third of the three waves of cell division which probably occur.

It is a pleasure to record my thanks for help in various aspects of this work, especially to Professor P. D. F. Murray and Dr. A. Glücksmann; to Professor M. M. Swann, and to Dr. D. N. Lawley for helpful suggestions in the statistical treatment. I should also like to express my appreciation of the financial help for this investigation from grants from the Scientific Investigations Grant-in-Aid administered by the Royal Society.

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Mesodermal Cell Deaths during the Early Development of the Scleral Bones in the Chick

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Summary

1. The positions of degenerating cells in the mesenchyme of the scleral bone primordia of the chick, and in the fibrous sclera beneath them, have been ascertained.

2. It is shown that the number of cell deaths within the bone primordia, and also beneath them in the fibrous sclera, is markedly higher than, and follows a different pattern from the regions between.

3. A centre of cell destruction is seen to occur at and near to the boundary of the mesenchyme and fibrous sclera. This begins in 7- to 8-day primordia, rises to a peak at 8 to 9 days, and subsides in 9- to 10-day primordia.

4. A second centre of cell death occurs in the mesenchyme nearer to the conjunctival papilla. It starts in 8- to 9-day eyes about mid-way between the conjunctiva and sclera, and as it increases in intensity, the centre moves nearer to the conjunctival papilla (9 to 10 days), beneath which it appears to subside (some 10-day primordia).

5. The function of these waves of cell destruction is discussed. It is concluded that their most likely function is morphogenetic; they may make a space into which the osteogenic cells migrate, or they may ensure a normal position and shape for the developing bone. Consideration has also been given to the possibility that the products of cell death might have a histiogenetic function.

Introduction

THE occurrence of cell deaths during normal embryological processes is an established but an unexpected phenomenon. The many isolated reports of cell degeneration during normal vertebrate ontogeny have recently been collated and surveyed by Glücksmann in a valuable review (1951).

Cell degeneration has been recorded in the development of cartilage or bone in only four instances. In the case of the skeleton of the limb, Fell (1925) has described cell deaths in the prechondral mass and surrounding tissue. Cell deaths also occur, not only in the thickened apical ridge of the limb buds, but also in the mesenchyme beneath it (Glücksmann, 1934). In the development of the sternum of birds, Fell (1939) has shown that intensive cell degeneration occurs at the same time as the migration of the sternal blastemas, which, originating near to the dorso-lateral body wall, can then move to their ventral positions, where the cartilages are laid down. Cell deaths also occurred in explanted sternal tissue. The fusion of the two halves of the sternum is made possible by the death of cells in the mid-ventral line. Jacobson and Fell (1941) showed that fusion of the two halves of the chick mandible took place in a similar way.

Jacobson and Fell also described how the angular, splenial, and supra-angular bones in the mandible of the chick are derived from a single condensation

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of mesenchyme. This condensation is divided into two as a result of cell de generation; one half forms the supra-angular and the other, subdivided b the mandibular nerve, the splenial and angular. Murray (1943), in his stud of the early development of the scleral bones in the chick, recorded death amongst the mesenchyme cells. These occurred among the transient collage fibres connecting the adjacent conjunctival papillae to the downwardly migra ing blastemas. It is interesting to note that Fell also found collagen fibre running among the degenerating cells in the development of the avian sternum

Murray (1943) did not make a particular study of cell degeneration in th mesenchyme. Therefore, while recording the positions of mitoses in this tissu (Hale, 1956), the positions of dying cells were also plotted, with the object o investigating more fully their possible role in the early development of th scleral bones.

An analysis of the occurrence of these cell deaths in the scleral bone pr mordia is presented in this paper. It shows that there is a very marked increas in cell deaths in the region where the mesenchyme merges with the fibrou sclera beneath it, and also in the mesenchyme nearer to the conjunctiva. It suggested that in both regions the cell deaths take place in waves of cell de struction, that at the mesenchyme–sclera boundary starting a little before tha in the mesenchyme. The possible function of these cell deaths is discussed.

Materials and Method

Twenty-three primordia were studied: six each from a 7-, 9-, and 10-da eye, and five from an 8-day eye. The material was the same as that in which th occurrence of mitoses was investigated, and the technical details will be foun in that paper (Hale, 1956). The incidence of cell deaths in the fibrous sclera, a well as in the mesenchyme above it, are included in the analysis.

Reconstructions of the positions of degenerating cells have been made i 'plan' and 'elevation' to give a three-dimensional picture of their distributio These reconstructions form the basis of quantitative analyses. Annuli wer drawn through the ring of primordia on the plan reconstructions, one for eac eye. Each annulus was then divided into segments of equal size, which were for all practical purposes, squares of side 0·125 mm. The number of ce deaths occurring in the pieces of tissue outlined by these segments wer analysed. For this purpose, the segments were segregated into those withi the bone primordia and those between the primordia. The delimiting c these 'within primordia' and 'between primordia' regions is the same a that described in my earlier paper.

In the following account a distinction is made between the mesenchym and sclera. The boundary between these two regions is often not very clear i embryos of these ages. In the majority of cases there is no difficulty in allocat ing a dying cell to one or the other tissue, but there are some near th boundary of the two where doubt exists. It will be seen that this ambiguity i not important in the present investigation.

Results

Cell deaths in the mesenchyme. In fig. 1 reconstructions are reproduced showing the distribution of cell deaths in the mesenchyme between the conjunctiva

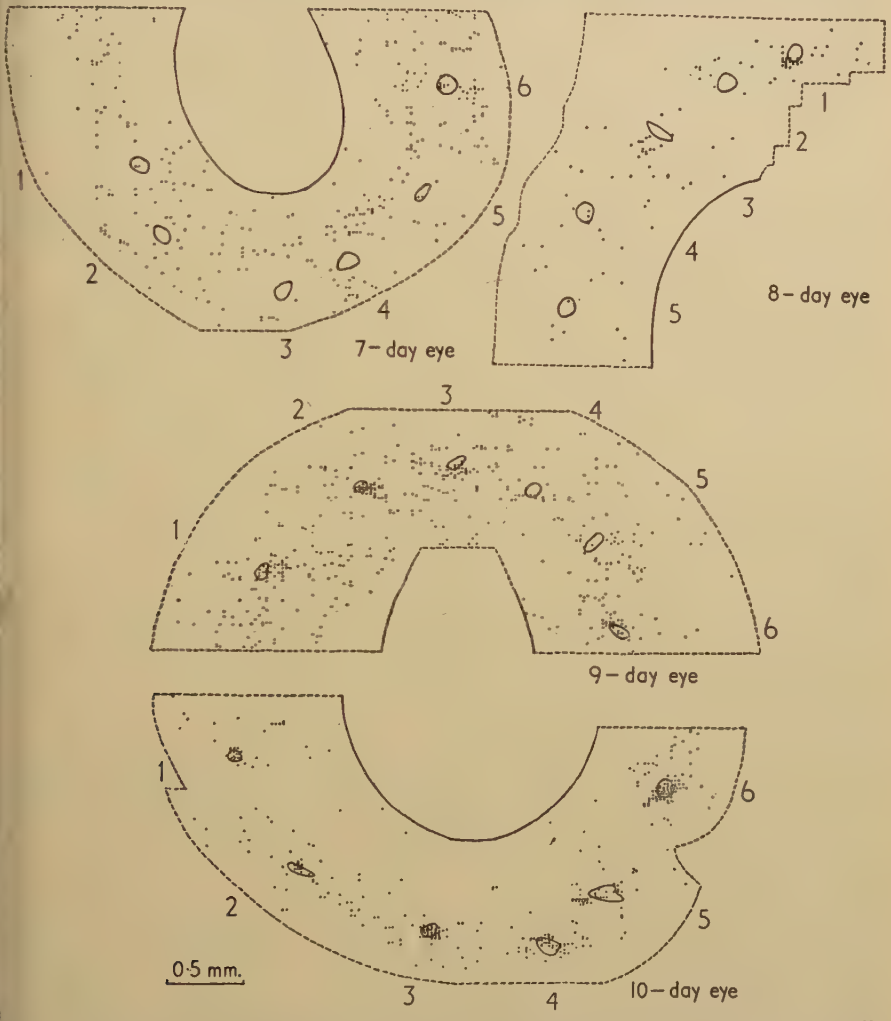

Fig. 1. Plan reconstructions of the cell deaths in the mesenchyme. Each reconstruction displays the dispositions of dying cells as if one were looking at the front of the eye. The inner full line marks the boundary of the anterior chamber of the eye, and the outer broken line shows where the eyelids (or nictitating membrane) are attached. The numbers refer to the primordia, the positions of which are indicated by the outline of the conjunctival papillae. Each dot represents one degenerating cell.

Note that below many of the older primordia there are numerous cell deaths.

above and the fibrous sclera below, and between the boundary of the anterior chamber of the eye and the base of the eyelids. This is the region in which the scleral plates appear.

In the 7-day eye cell deaths are seen to be fairly numerous; but with the possible exception of primordium No. 6, there is little distinction between the numbers found beneath the conjunctival papillae and the numbers found elsewhere. In primordium No. 6 there is a suggestion of a small group of degenerating cells. In the 8-day eye primordium No. 1 has a marked group of degenerating cells, and there may also be a group in No. 3. The number of cell deaths in the mesenchyme generally is less than in the 7-day eye. Groups of dying cells are found in four of the six 9-day primordia (Nos. 1, 2, 3, and 6); the other two primordia cannot be distinguished from the rest of the mesenchyme. The latter has once again a higher incidence of cell deaths. All the primordia in the 10-day eye are conspicuous by their content of dying cells; the distinction appears to be even more clear since there are again few cell deaths in the surrounding mesenchyme.

Thus, as the primordia become older, it appears that more cell deaths occur within them. This is confirmed by fitting a regression line to the number of cell deaths in the 'within primordia' segments of the annuli, the age of the embryo being used as the independent variable. Such a line has a slope differing significantly from zero ($t = 4{\cdot}81$, D.F. $= 55$, and $P < 0{\cdot}001$).

It has been shown (Murray, 1943; Hale, 1956) that the cell population density also increases, both within and between primordia, and thus more cell deaths might be expected, without the increase being significant. The cell population density has therefore been combined with the number of degenerating cells in order to estimate a third parameter, which, while still measuring degenerative activity, eliminates the effect of changing cell population density. This third measure is called the 'degeneration index', and is the number of degenerating cells per 1,000 cells. The degeneration index has been calculated for each segment of the four annuli.

Similar results are obtained from the analysis of the degeneration index as from the analysis of the total number of cell deaths. A regression line fitted to the degeneration index within the primordia, with the age of the embryo as the independent variable, shows a significant rise with increasing age ($t = 5{\cdot}46$, D.F. $= 55$, and $P < 0{\cdot}001$).

The frequency distribution of the number of cell deaths in the 'between primordia' segments of the annuli of the four eyes is found to be adequately described by the terms of a negative binomial (modified Poisson) distribution. This distribution was chosen because it seemed possible that the expected number of cell deaths in the annuli might not be constant: owing to the uncertainty in defining the limits of a primordium in some cases, the expected number of cell deaths in a 'between primordia' segment adjacent to a 'within primordia' segment might be different from other 'between primordia' segments. The test of 'goodness of fit' of the distribution gives no evidence for departure from randomness of the data ($\chi^2 = 0{\cdot}75$, D.F. $= 4$, and $P \simeq 0{\cdot}85$).

In table 1 is recorded the maximum number of cell deaths found in a segment of an annulus within a primordium. The corresponding degeneration index is also included. The distribution above indicates that a number of cell

deaths of 6 or more has a probability of occurrence of 0·02. Those primordia having a level of cell destruction greater than can be accounted for by random fluctuations (at this level of significance) are indicated in the table by an asterisk.

Table 1 shows that, as the age of the eye increases, more and more primordia have a significantly high incidence of cell deaths, when the frequency of occurrence between the primordia is used as a basis for comparison.

TABLE 1

Level of cell destruction within individual primordia

Age of eye (days)	*Primordium No.*	*Number of cell deaths*	*Degeneration index*	*Age of eye (days)*	*Primordium No.*	*Number of cell deaths*	*Degeneration index*
7	1	2	1·6	9	1	14*	5·2
	2	1	1·0		2	25*	11·8
	3	0	0·0		3	14*	5·6
	4	2	0·9		4	6*	4·5
	5	2	0·9		5	5	1·7
	6	7*	4·2		6	14*	4·4
8	1	13*	6·1	10	1	15*	8·3
	2	1	0·5		2	13*	5·5
	3	9*	5·8		3	29*	12·5
	4	2	1·0		4	18*	8·9
	5	1	0·8		5	9*	6·2
					6	29*	13·3

* Indicates a significantly high level of cell destruction ($P \leqslant 0{\cdot}02$).

Cell deaths in the fibrous sclera. If we turn now to the distribution of cell deaths in the fibrous sclera, which lies beneath the mesenchyme region (that is, nearer to the retina), fig. 2 indicates that the number of dying cells immediately beneath the bone primordia is not very different from other regions in the sclera. In the 7-day eye there is some suggestion of more dying cells in the region of Nos. 1, 2, and 4, and again in No. 4 of the 8-day eye. In the 9-day eye it is difficult to pick out primordia which show more dying cells in the sclera beneath them. Cell deaths are more or less uniformly distributed through the sclera of the 10-day eye. The large numbers of cell deaths near the boundary of the anterior chamber of the eye occur in the ciliary muscle primordium.

When a regression line is fitted to the numbers of cell deaths in the regions of the sclera beneath the primordia, with the age of the embryo as the independent variate, it is found that the number of cell deaths probably decreases significantly ($t = 1{\cdot}94$, D.F. $= 55$, and $P \simeq 0{\cdot}05$). In the other parts of the sclera there is also a significant decrease with increasing age of embryo ($t = 2{\cdot}38$, D.F. $= 74$, and $P \simeq 0{\cdot}02$). The data do not reveal a difference between the rates of decrease ($t = 0{\cdot}42$, D.F. $= 129$, and $0{\cdot}7 > P > 0{\cdot}6$), but the level of cell destruction is almost certainly higher in the regions

beneath the primordia (4·2) than in the regions between (2·9) ($t = 2{\cdot}62$, D.F. = 131, and $P \simeq 0{\cdot}01$).

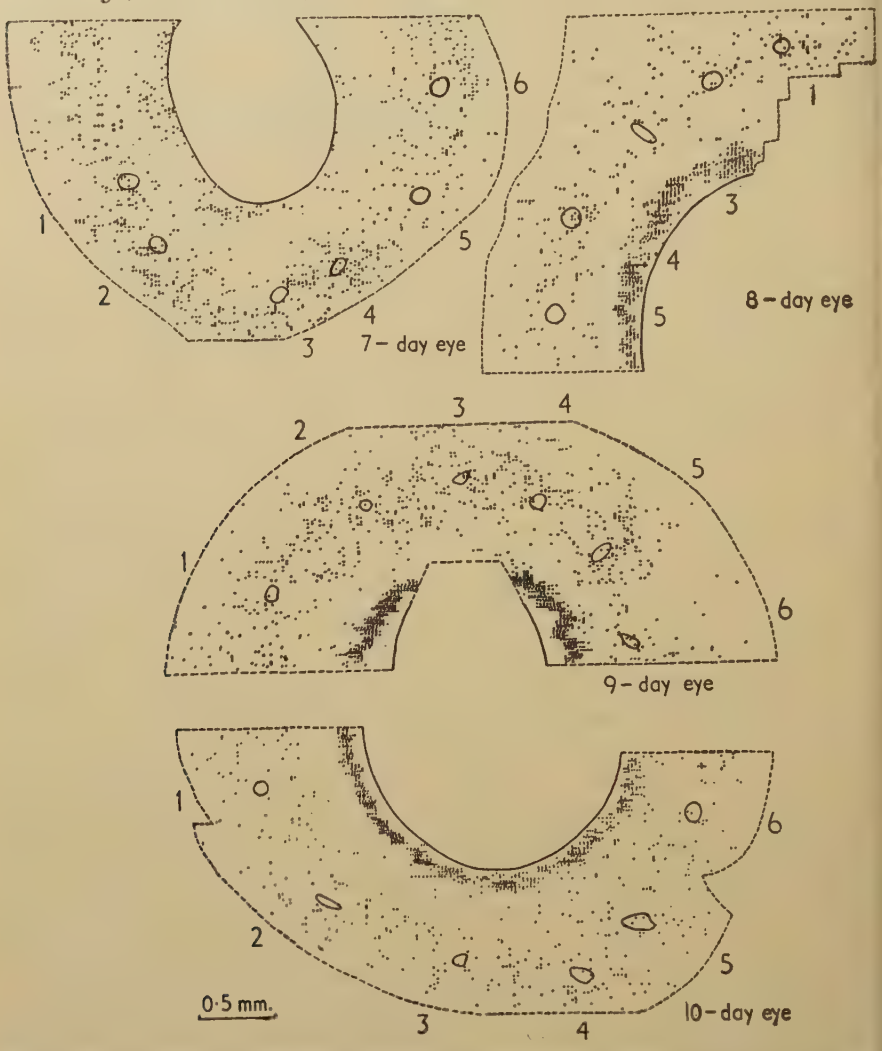

FIG. 2. Plan reconstructions of the cell deaths in the sclera. The cell deaths in the sclera are reconstructed in a similar way to those in the mesenchyme (fig. 1).

The regions of the sclera below the conjunctival papillae are in no case very conspicuous by any greatly increased numbers of cell deaths, but quantitative tests do reveal a greater numbe in some cases (see text).

The large numbers of cell deaths near to the boundary of the anterior chamber of the eye occur in the ciliary muscle primordium.

Day-to-day changes in the number of cell deaths are recorded in table 2 together with tests to determine whether these changes are greater than could be accounted for by random fluctuations.

In the regions beneath the primordia, and also in the regions between, th

level of cell death is highest at 7 and 9 days, with a marked drop at 10 days of incubation. Whereas all the changes are probably significant between the primordia, beneath the primordia there is no support for the apparent decrease from 7 to 8 days. The highest number of cell deaths beneath the primordia occurs at 9 days.

TABLE 2

Daily changes in the number of cell deaths in the sclera

	Age of eye (days)	Number of cell deaths in segments of annuli			Comparison between successive days		
		Mean	Standard error of mean	Number of observations	t	D.F.	P
Beneath primordia	7	3·9	±0·66	23			
					2·35	35	$0{\cdot}05>P>0{\cdot}02$
	8	2·1	±0·27	14			
					3·37	29	$0{\cdot}01>P>0{\cdot}001$
	9	3·6	±0·35	17			
					2·06	35	0·05
	10	1·9	±0·43	22			
Between primordia	7	5·0	±1·02	16			
					0·75	26	$0{\cdot}5>P>0{\cdot}4$
	8	4·0	±0·90	12			
					2·00	24	0·05
	9	6·1	±0·58	14			
					6·59	27	<0·001
	10	1·7	±0·36	15			

The elevation distribution of cell deaths. The foregoing analyses have demonstrated beyond reasonable doubt that the numbers of cell deaths within the scleral bone primordia and in the fibrous sclera beneath the primordia are at a different level, and follow a different pattern from the numbers found between the primordia. Within the mesenchyme of the primordia, the numbers rise to a maximum at 10 days of incubation, and in the sclera beneath the greatest degenerative activity occurs at 9 days. From the elevation reconstructions (a selection of which is reproduced in fig. 3), these cell deaths do not appear to be distributed equally at all distances from the conjunctiva. If there were no difference, the same frequency of cell deaths (within sampling variations) would be found at all distances. Taking the mean number of cell deaths at all distances as this expected frequency, the observed frequencies differ too much to accept them as random variations (mesenchyme: $\chi^2 = 346$, D.F. = 19, and $P < 0{\cdot}001$; sclera: $\chi^2 = 93$, D.F. = 11, and $P < 0{\cdot}001$).

The possibility that the variation in the number of cell deaths at different distances from the conjunctiva conforms to an organized pattern was investigated. Within an embryo, the primordia are not all at precisely the same stage of development, although they are technically of the same age. Although, in general, the primordia of one eye are less advanced than in an older eye, this variation can be such that a more advanced primordium in one eye can be at the same stage of development as a less advanced primordium in an embryo

incubated a day longer. It is therefore possible to obtain a more detailed time series from the study of individual primordia, the age of the embryo being used as a broad guide. From such a study, it is possible to arrange the primordia in a sequence which shows a wave of cell destruction in the mesenchyme, and at the same time also shows a second wave in the region of the mesenchyme-sclera boundary. In this reconstruction of events, no upward trend

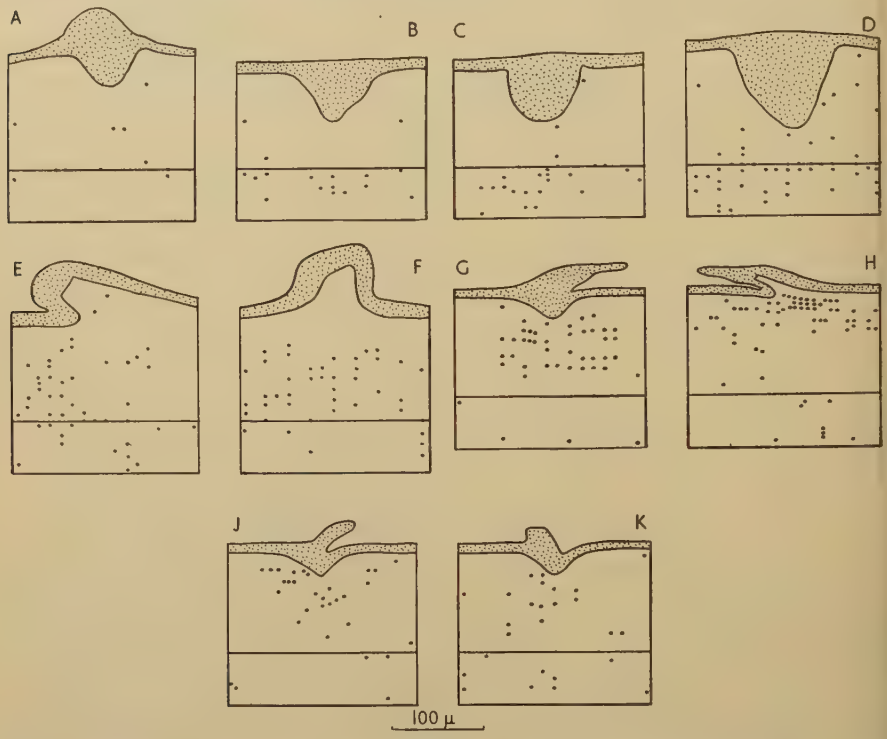

FIG. 3. Elevation reconstructions of cell deaths. Each diagram is a reconstruction of cell deaths in the mesenchyme of a primordium, and in the fibrous sclera beneath it. Each degenerate cell is indicated by a dot. The conjunctival papilla is also shown (stippled). A, 7 days, No. 5; B, 8 days, No. 2; C, 8 days, No. 4; D, 9 days, No. 4; E, 9 days, No. 6; F, 9 days, No. 1; G, 10 days, No. 3; H, 10 days, No. 6; J, 10 days, No. 1; K, 10 days, No. 2. Note the increase and decrease in the number of cell deaths at the mesenchyme–sclera boundary (B–F), and in the mesenchyme (D–K). The centre of the region of cell deaths in the mesenchyme moves from the middle of the mesenchymal region (E, F) to just beneath the conjunctival papilla (H), before subsiding (J, K).

appears in the mesenchymal wave until 9 days of incubation, and the peak of this degenerative wave is reached in some 10-day primordia. During this time the centre of cell destruction becomes progressively nearer the conjunctival papilla. In two 10-day primordia (Nos. 1 and 2) the centre of cell destruction is near the papilla, but they have a markedly reduced number of cell deaths as compared with other 10-day primordia; this is taken to indicate a subsidence of the wave of cell degeneration. The earlier stages in the wave of cell death at the mesenchyme–sclera boundary appear in 7- and 8-day primordia, the peak

is reached in some 9-day primordia, and cell destruction decreases again in other 9-day and in 10-day primordia.

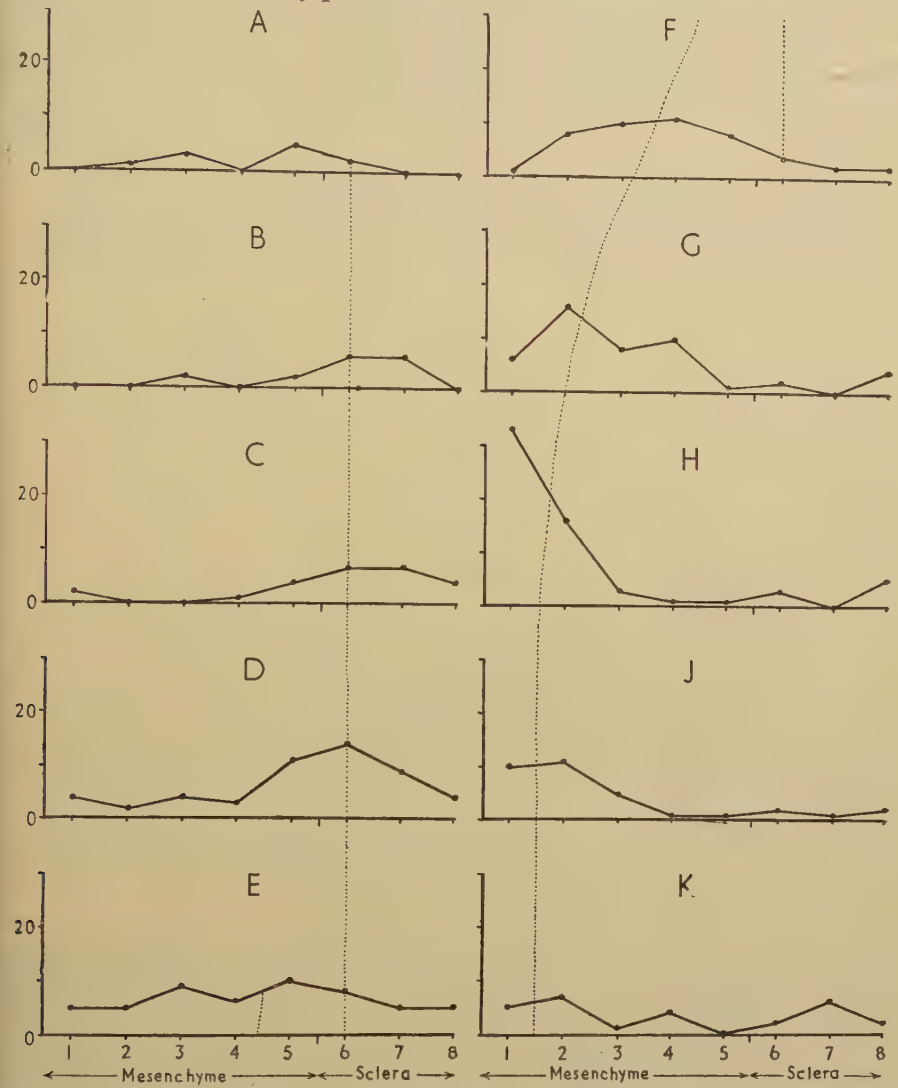

FIG. 4. Graphical illustration of the elevation distribution of cell deaths. The mesenchymal region is divided into five, and the sclera into three slices parallel to the conjunctiva, the slices being approximately equal in size. The numbers of cell deaths in the slices (ordinates) are plotted against the corresponding distances of the slices from the conjunctiva (abscissae). The conjunctiva is on the left, and the bottom of the sclera on the right. These graphs illustrate the elevation distribution of cell deaths in the primordia illustrated in fig. 3.

The increase and decrease in the number of cell deaths at the mesenchyme–sclera boundary and in the mesenchyme is shown by the dotted lines.

These postulated waves of cell death are illustrated in fig. 3 by a selection from the elevation reconstructions. The number and dispositions of cell deaths in these primordia are also shown in a series of graphs in fig. 4. In the graphs

the mesenchymal region is divided into five, and the sclera into three slices parallel to the conjunctiva, all the slices being approximately equal in volume. The number of cell deaths in these slices is plotted against their distance from the conjunctiva. The waves of cell deaths are shown by the formation and subsidence of peaks in the curves at the mesenchyme–sclera boundary and in the mesenchyme.

Further illustration of these suggested waves is provided by fig. 5, which includes all the data from all the primordia studied. The succession of primordia is assumed to illustrate the time sequence (abscissae), and the number of cell deaths are the corresponding ordinates. Since one wave appears to affect

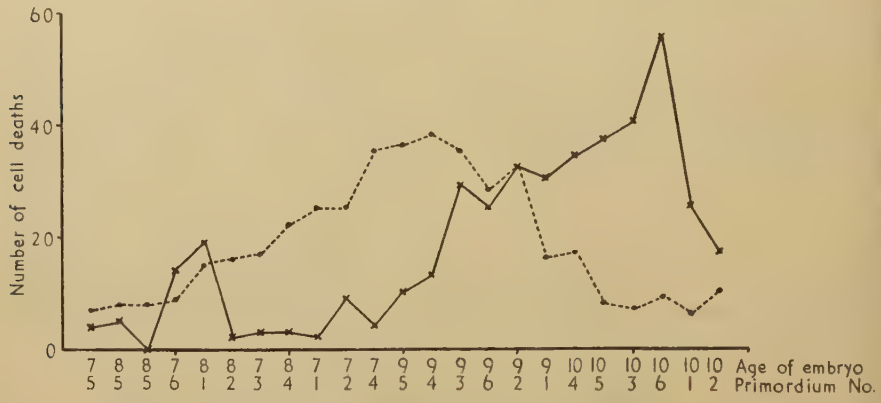

Fig. 5. Graphical illustration of the suggested waves of cell deaths. The 23 primordia have been arranged in an order which is assumed to represent the time sequence (abscissae); the numbers of degenerate cells in the upper four-fifths of the mesenchyme (full line), and the rest of the mesenchyme plus the sclera (broken line) in each primordium are the corresponding ordinates.

In the mesenchyme there is a rise in cell destruction to a marked maximum in older primordia, and a small unexplained 'hump' due to two young primordia. Note also the rise and fall in the number of cell deaths in the sclera plus the lowest fifth of the mesenchyme; this occurs earlier, but overlaps the wave of cell deaths in the mesenchyme.

both mesenchyme and sclera, where they are adjacent to one another, the data have been redivided; the number of cell deaths in the upper four-fifths of the mesenchyme (i.e. nearer to the conjunctiva) being used to demonstrate the wave in the mesenchyme, and the number of cell deaths in the rest of the mesenchyme plus the sclera to show the wave at the mesenchyme–sclera boundary.

These graphs show clearly the rise and fall of the number of cell deaths in the mesenchyme–sclera boundary region; and also, as this wave reaches its peak, the rise in the incidence of cell deaths in the mesenchyme takes place. The latter graph also shows a small but marked peak due to two younger primordia (7-day, No. 6; and 8-day, No. 1). Although it is difficult to test adequately whether the number of cell deaths in these two primordia are random fluctuations, the numbers appear to be rather large for this explanation. I am unable to suggest an explanation of their occurrence.

Fig. 6 illustrates more fully the location of these cell deaths. The abscissae are the distances from the conjunctiva, the conjunctiva being on the left, the bottom of the sclera on the right, and the junction of the mesencyhme and sclera between the two. The ordinates are the total number of cell deaths in all the primordia at the various distances. The peak in the mesenchyme and that at the mesenchyme–sclera boundary are readily noticeable.

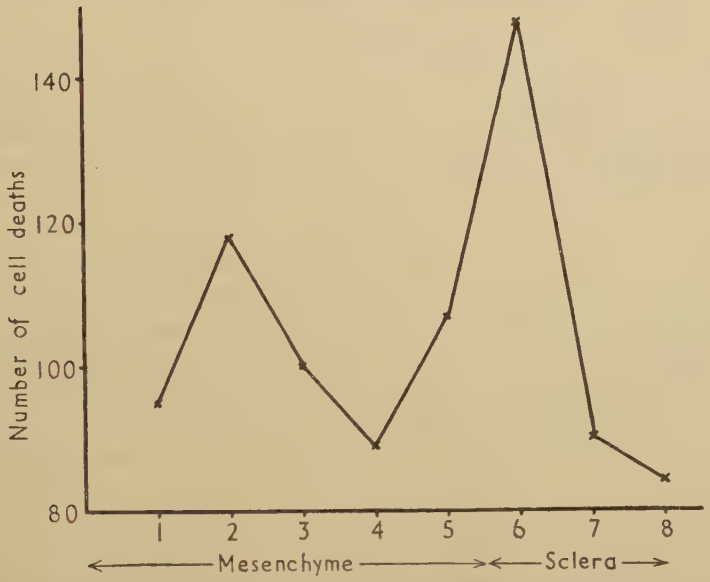

Fig. 6. The position of the suggested waves of cell deaths. The abscissae are the distances from the conjunctiva of the eight slices of tissue referred to in fig. 4; the conjunctiva is on the left, and the bottom of the sclera on the right. The ordinates are the corresponding numbers of cell deaths in these slices.

Note the two maxima on the curve; one occurring in the mesenchyme, and the other at the top of the fibrous sclera.

These waves of cell deaths are postulated partly on the basis of empirical observation of the reconstructions of cell deaths in individual primordia, and it may be asked what evidence there is to support these suggestions. Quantitative analyses have shown, first, that the incidence of cell deaths within the primordia and in the sclera beneath the primordia, is higher than, and follows a different pattern from the corresponding tissues between the primordia. Secondly, both within the primordia and beneath them in the sclera, more cells die at some distances and less at other distances than can be accounted for by random variation. The only reasonable deduction from these facts is that it is probable that the cells degenerate in conformity to some organized pattern. There can be little doubt that there are two centres of cell destruction (fig. 6). The simplest and most reasonable expectation (in each case) is an increase to a peak of degenerative activity, followed by a decrease. The centre of cell destruction at the mesenchyme–sclera boundary is constant in position. The focus of cell deaths in the mesenchyme, however, may be anywhere from

the middle of this region to just beneath the papilla; and the most likely explanation of this is that it moves from the centre towards the papilla, or in the reverse direction. It should also be noted that stages in the two suggested waves occur together in about a third of the primordia studied. To incorporate these facts and suggestions within the broad time-scale of the age of the embryo virtually precludes any explanation other than the one I have suggested.

Discussion

The cell deaths recorded in this investigation undoubtedly correspond to those seen by Murray (1943) amongst the fine collagen fibres running downwards towards the sclera from the base of the conjunctival papilla. The degenerative phase is seen, however, to be more extensive, and to affect cells at the mesenchyme–sclera boundary, as well as mesenchyme cells nearer to the conjunctiva.

The relation of cell deaths to other known phenomena. (*a*) *Cell division.* Cell division in the bone primordia has been seen to take place mainly near the conjunctiva (Hale, 1956). There are probably three waves of cell division of decreasing intensity: the first occurring in young 7-day primordia, the second in 7- and 8-day primordia, and the third in 9- and 10-day primordia. There is also evidence that the cells of the primordium continue to divide as they migrate to their definitive position near the sclera (10-day primordia). The wave of cell destruction taking place near to the mesenchyme–sclera boundary thus occurs at some distance from the centre of cell division, and when the number of mitoses has decreased considerably. The upwardly moving wave of mesenchymal cell deaths takes place towards the end of the downward migration of the primordial bone cells, and thus to the movement towards the sclera of the dividing cells. There seems little likelihood, therefore, that the cell deaths are closely linked to cell division.

(*b*) *Cell deaths in the conjunctival papilla.* Murray has shown that cell deaths in the conjunctival papilla occur in two overlapping stages. In the first, some periderm cells degenerate (7 to 8 days); and in the second, cells in the body of the papilla degenerate (8 to 9 days), later becoming so extensive (9 to 10 days) that the papilla eventually shrinks to become a thin solid projection (10 days), which dwindles and disappears (10 to 12 days). Ranking analyses have shown that in the 7- and 8-day primordia, there is no evidence of a correlation between ectodermal and mesodermal degeneration ($t = 1{\cdot}11$, D.F. $= 9$, and $0{\cdot}3 > P > 0{\cdot}2$). But in the 9- and 10-day eyes, a ranking analysis shows a high degree of correlation ($t = 5{\cdot}50$, D.F. $= 10$, and $P < 0{\cdot}001$), giving evidence of some relationship between the two sites of cell death.

(*c*) *Cell population density.* It is possible that the considerable increase in the cell population density in older bone primordia might give rise to a condition of anoxia, shortage of food materials, or accumulation of excretory products, and this might be the cause of the increased number of cell deaths. Ranking analysis of the 9- and 10-day primordia gives no support to this suggestion ($t = 0{\cdot}41$, D.F. $= 10$, and $0{\cdot}7 > P > 0{\cdot}6$).

(*d*) *Cell migration.* Murray showed that the cells of the mesenchyme condensations, originally near the conjunctival papillae (7–8 days), move away from them towards the fibrous sclera (9 days). Then, just above the fibrous sclera, the condensations flatten out (10th day), and it is here that the bones begin to form. It will be seen that cell destruction takes place at the same time as cell migration. Cell deaths at the junction of the fibrous sclera and mesenchyme occur as the cells of the condensations move towards the sclera, and the wave of cell destruction in the mesenchyme coincides with the later stages of migration, and with the flattening out of the condensations.

The cause of cell deaths. This investigation has provided no clue as to the cause of the cell deaths.

The function of cell destruction. Glücksmann (1951) has distinguished three main functions of cell degeneration: morphogenetic, histiogenetic, and phylogenetic. It is instructive to consider the function of the cell deaths described above in the light of his classification.

(*a*) *Morphogenetic function.* Morphogenetic cell destruction can make the space necessary for the movement of cells from one place to another, as, for instance, has been shown by Fell (1939) to be the case in the migration of the sternal rudiments in birds. In the case of the scleral bone primordia, the cell deaths at the mesenchyme–sclera boundary might well be making a space for the downwardly migrating cells of the condensations, and thus have a morphogenetic function.

The cell deaths in the mesenchyme, the focus of which moves towards the conjunctiva, could hardly have such a function. They occur during the later migration stages, in the place from which the cells have moved. Also, the centre of cell destruction moves in the opposite direction to that of the osteogenic cells.

Cell destruction might have a shaping function. Perhaps the destruction of cells above and below the primordium makes a contribution to the flattening process.

Towards the end of the wave of cell degeneration in the mesenchyme, deaths are noted near the conjunctival papilla. Their positions suggest a sort of 'mopping up' operation; stragglers in the movement of the condensation being removed, so that the production of a bone, normal in position and shape, is assured. Many cases of the destruction of superfluous cells during embryonic processes are known, but these are concerned with the separation or junction of organs, or the hollowing out of originally solid rudiments.

(*b*) *Histiogenetic function.* Murray related these cell deaths to the formation of the transient collagen fibres which grow down from the base of the conjunctival papilla as the condensation moves downwards. Fell (1939) also reported collagen fibres amongst degenerating cells during the migration of the sternal rudiments of birds. The present investigation has shown that the wave of cell destruction in the mesenchyme, although occurring at the same time as the growth of the fibres, moves in the opposite direction. It is difficult to see any connexion between fibre growth and the cell deaths at the mesenchyme–

sclera boundary, as the fibres never grow as far as this. Cell deaths and fibre formation do not therefore seem to be closely related.

Brachet (1947) has suggested that ribonucleic acid, liberated by the death of cells, might have an evocatory function. His theory appears to be supported by the recent work of Perri (1951), who produced supernumerary limbs in *Bufo vulgaris*, which, he suggests, is due to the production of ribonucleic acid, or its destruction products, by the X-rayed tissue he grafted on to the experimental animals. In the case of the scleral bones, cell destruction takes place in the conjunctival papillae at the same time as in the mesenchyme beneath; and it is not unlikely that at this time the cells of the scleral bone primordia become determined as osteoblasts. The cell deaths might therefore have a histiogenetic function.

(*c*) *Phylogenetic function.* By phylogenetic cell death is meant the destruction of some, now lost, ancestral structure. There is no known structure in this position in the ancestors of birds, and it is difficult to conceive of any.

It is a pleasure to record my thanks for help in various aspects of this work, especially to Professor P. D. F. Murray and Dr. A. Glücksmann, to Professor M. M. Swann, and to Dr. D. N. Lawley for helpful suggestions in the statistical treatment. I should also like to express my appreciation of the financial help for this investigation from grants from the Scientific Investigations Grant-in-Aid administered by the Royal Society.

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The Morphology and Origin of the Golgi Bodies and their Role in the Secretion of the Acrosome in the Spermatogenesis of Pulmonate Gastropods as Determined in the Living Material by Phase-contrast Microscopy

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With four plates (figs. 1–4)

Summary

In this paper are embodied our observations on the morphology, origin, and role of the Golgi bodies in the spermatogenesis of the slug, *Anadenus altivagus*, and the snail, *Euaustenia cassida*. Living cells have been studied under the phase-contrast microscope and photomicrographed. In the juxta-nuclear Golgi region in primary spermatocytes the Golgi bodies exist in the form of (1) granules and rods of dark contrast, (2) spheres of pale contrast, and (3) spheroids showing a duplex structure, each consisting of a sphere of pale contrast and an incomplete or complete cortex or sheath of dark contrast. The Golgi spheroids have been shown to arise three times in the course of spermatogenesis from the mitochondrial granules by a process of alignment. The acrosome is formed from a cap of tiny acrosomal granules, which are deposited in front of the spermatid nucleus by the Golgi bodies (acroblasts).

Introduction

IN a remarkable paper Roque (1954) has studied the morphology of the living spermatocytes and spermatids (and also neurones) of *Helix* as revealed by phase-contrast and interference microscopy and by supra-vital dyes. Roque gives also a very useful, indeed masterly, review of the previous literature on the spermatogenesis of the pulmonate gastropods, to some of which references will be made in the body of this communication. But he does not quote the paper of Hickman (1931) on the spermatogenesis of the pulmonate, *Succinia ovalis*, and particularly of Watts (1952), who examined the spermatogenesis in the slug, *Arion subfuscus*, with phase-contrast microscopy.

Roque (1954) has given a very detailed and a remarkably accurate account of the morphology of the Golgi bodies (his paranuclear bodies), but he is doubtful about their origin. Nor did Roque find any evidence 'of a direct participation of the paranuclear bodies in the formation of the acrosome'.

Nath (1955) and Nath and Chopra (1955) have published two papers on the spermatogenesis of the Simla slug, *Anadenus altivagus*. These authors have used exclusively the Flemming-without-acetic and iron haematoxylin technique. They have shown that the Golgi dictyosomes take their origin from the mitochondria three times in the course of spermatogenesis (having merged into them twice) and that the acrosome is formed from the acrosomal granules, which in the first instance are secreted in the chromophobe sphere enclosed by the U-shaped acroblast (Golgi dictyosomes).

[Quarterly Journal of Microscopical Science, Vol. 97, part 3, pp. 369-377, September 1956.]

Nath (1956) has stated that the Flemming-without-acetic acid and iron haematoxylin technique introduces no artifacts, and such preparations are even more valuable in the study of spermatogenesis than those of living cells because they are stained and permanent, thus enabling the details to be worked out at leisure.

In this paper are embodied our observations on the living cells as seen under phase-contrast in the spermatogenesis of the Simla slug, *A. altivagus*, and of the snail, *Euaustenia cassida*, obtained from Bharwain, which is at the distance of about 27 miles from Hoshiarpur at an altitude of about 3,000 feet above sea-level. *Anadenus* was studied by the authors on the spot (Simla) in August 1955, and living specimens of *Euaustenia*, collected at Bharwain by Mr. Kuldip Chand, were studied at Hoshiarpur in October 1955.

Carl Zeiss Stand 'W' phase-contrast microscope was used in our studies. Very small pieces of the ovotestis were studied in 0·7% NaCl solution, to every 100 cc of which 0·2 ml of 10% $CaCl_2$ had been added (Baker, 1949). The margins of the coverslip were sealed with vaseline to prevent evaporation. For supra-vital staining with neutral red chloride (B.D.H.), 0·01% of the dye was dissolved in the above-mentioned physiological solution. Both positive phase-contrast and ordinary microscopy were used for studies with this dye.

Photomicrographs were taken with a Carl Zeiss micro-reflex camera attachment and Contax camera, on Kodak Pan-X 35 mm film. All the photomicrographs were taken with the 1·25/100 oil-immersion objective and K 8X ocular. They were further enlarged 3 or 4 times and were untouched. The exposure times were standardized at 1 second.

Observations

Spermatogonia

Fig. 1, A is a photomicrograph of a spermatogonium of *Anadenus*. The mitochondria are arranged in the form of a juxta-nuclear mass, consisting of granules and filaments. In both *Anadenus* and *Euaustenia* there are no traces of Golgi bodies in the spermatogonia.

Primary spermatocytes and first meiotic division

In the early growth period of the primary spermatocyte, some of the mitochondrial granules begin to grow and give a phase-change higher than the rest of the mitochondria. These darker granules begin to align themselves and form very short rods, which have a crenated appearance. These are the precursors of the Golgi spheroids (figs. 1, B and 3, A, B).

With the growth of the primary spermatocyte the crenated short rods begin

Fig. 1 (plate). Photomicrographs of living male germ cells of *Anadenus altivagus* under positive phase-contrast (except H, which was photographed by direct microscopy).

A, spermatogonia showing juxta-nuclear mass of mitochondria.

B, early primary spermatocyte, showing the crenated rods formed by the alignment of mitochondrial granules.

C to G, primary spermatocytes, showing the different forms of Golgi bodies.

H, primary spermatocyte stained supravitally with neutral red chloride.

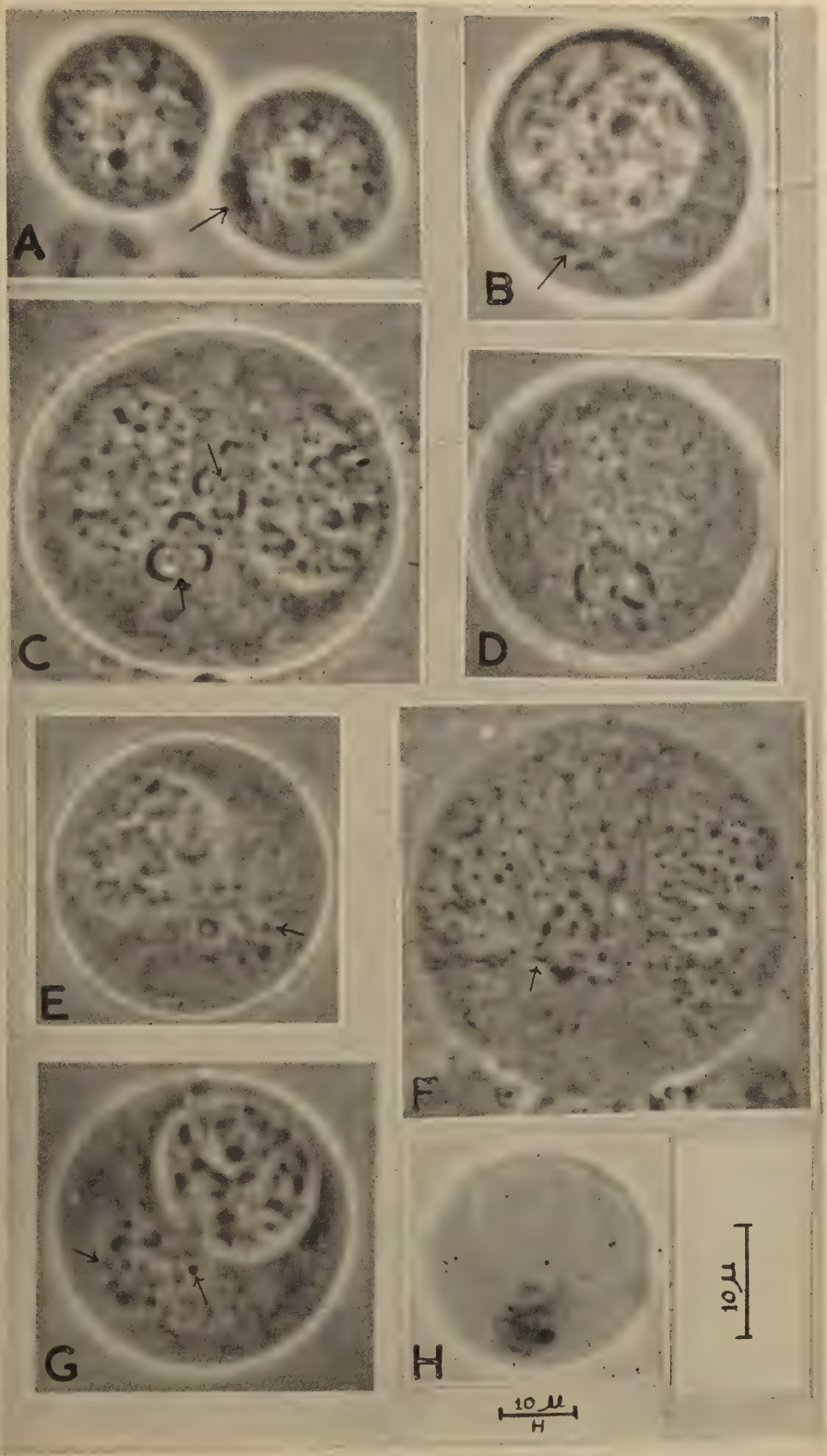

Fig. 1

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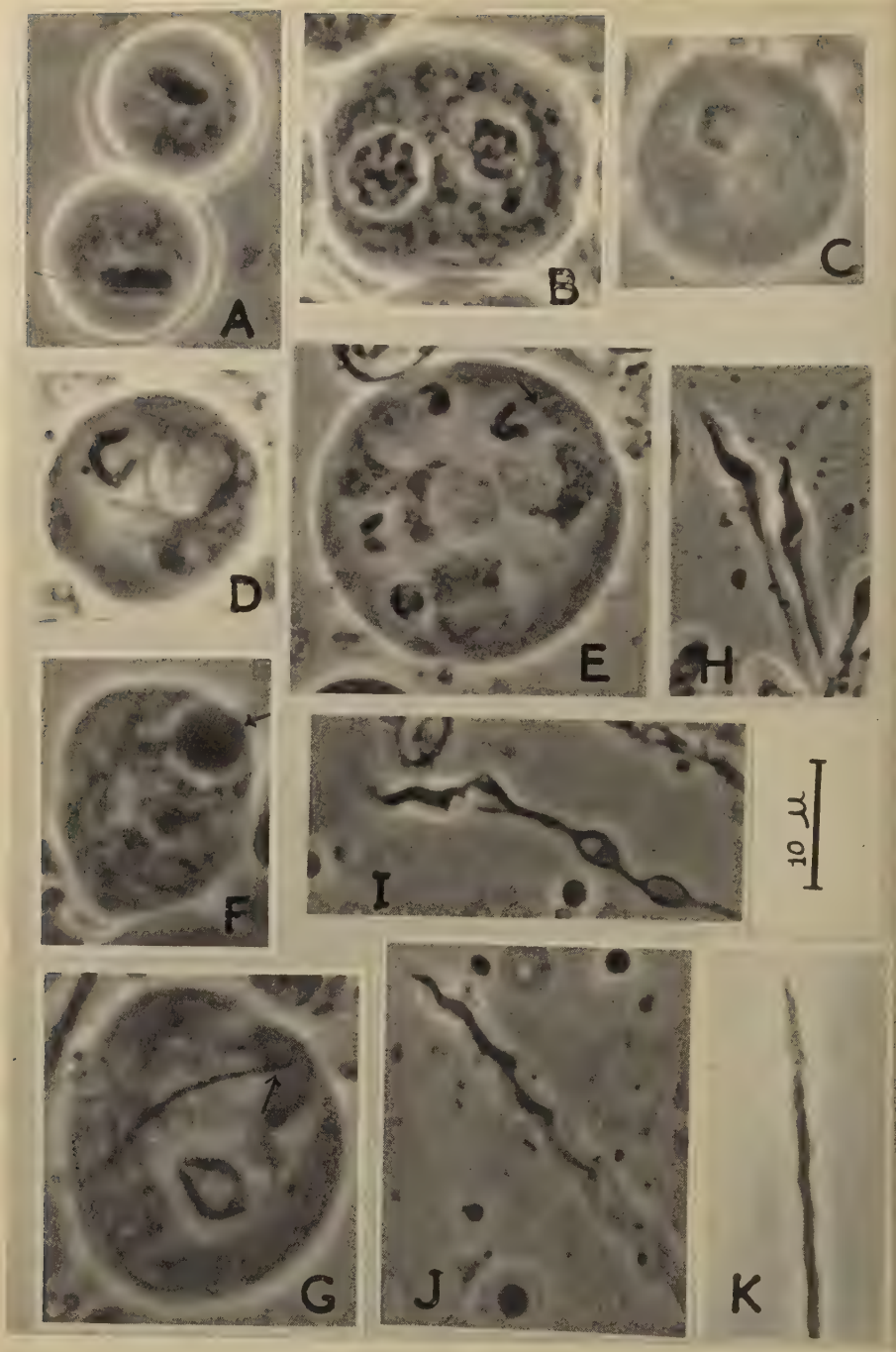

FIG. 2

V. NATH *and* B. L. GUPTA

to grow and acquire an absolutely smooth contour. Ultimately they take up a position in the cell near the nucleus. At the same time several of these rods develop each a clear sphere in close association with them, the material of each rod gradually extending itself in the form of an incomplete or complete cortex over the sphere. Thus are formed the Golgi spheroids with a duplex structure, each consisting of a clear sphere, giving a low phase-change, and a very dark shining cortex, giving a very high phase-change.

An extensive study of this juxta-nuclear collection under phase-contrast in living, fully mature primary spermatocytes clearly shows that there are at least six kinds of elements in this collection: (1) granules, (2) rods, (3) rods associated with spheres, (4) spheres with incomplete cortices, (5) spheres with complete cortices, and (6) spheres only.

The granules in this collection give a very high phase-change, and can be clearly seen in fig. 1, C, G. Sometimes one comes across rods only, which give an equally high phase-change (fig. 1, D). These rods, like the granules, are not very common; in fact they may be said to be rather rare in mature spermatocytes. But the spheres with incomplete and complete cortices are commonly met with. When the cortex of the sphere is complete, the element will appear in the form of a ring in optical sections at certain foci (figs. 1, E and 3, D); but when the cortex is incomplete the element may appear as a crescent with or without the sphere (figs. 1, C and 3, C) or as a rod with tapering ends (figs. 1, F and 3 D), depending on the position and the focus of the element. It may be mentioned here that the spheres with sheaths are, to begin with, clear and vacuole-like (fig. 1, C), but after some time they look dense, giving a greyish contrast. Lastly, the elements in the juxta-nuclear collection may be in the form of dense spheres only, giving a greyish contrast (figs. 1, E–G and 3, C).

It may also be mentioned that the Golgi spheroids of *Anadenus* are distinctly bigger than those of *Euaustenia*, and that in *Anadenus* the 'rings' are rather rare as compared with the latter species.

We were not fortunate enough to obtain stages of the first meiotic division in *Anadenus* in the month of August, when the ovotesticular material of this species was studied at Simla, but we obtained a good photomicrograph of telophase I in *Euaustenia* in October at Hoshiarpur (fig. 3, E). In this figure the Golgi elements which had presumably merged into the mitochondria during the earlier metaphase I (see Nath, 1955, and Nath and Chopra, 1955, on

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FIG. 2 (plate). Photomicrographs of living male germ cells of *Anadenus altivagus* under positive phase-contrast.

A, telophase II, showing the absence of Golgi bodies.

B, late telophase II, showing the reappearance of Golgi bodies by alignment from mitochondrial granules.

C, early spermatid showing three Golgi dictyosomes enclosing a sphere between them.

D, spermatid showing acrosomal material flowing out of the acroblast.

E, spermatids (syncytium). The acrosomal granules are coming out of the acroblast in the form of a white streak.

F to J, maturing spermatids.

K, mature spermatozoon.

Anadenus) are once more just beginning to arise from the mitochondria, as is clear from the fact that some mitochondrial granules have just begun to acquire a higher phase-change, till in the secondary spermatocytes (fig. 3, F, G) Golgi spheroids of characteristic form are formed.

Secondary spermatocytes and second meiotic division

Thus it is clear that in the secondary spermatocytes (fig. 3, F, G) the Golgi spheroids take their birth from the mitochondrial granules for the second time in the course of spermatogenesis.

Nath (1955) and Nath and Chopra (1955) state that during metaphase II and anaphase II in *Anadenus*, the Golgi bodies again break up to merge into the mitochondrial granules, to reappear again during late telophase II. In our studies with phase-contrast microscopy we have been fortunate to photomicrograph metaphase II in *Euaustenia* (fig. 3, H) and early and late stages of telophase II in *Anadenus* (fig. 2, A, B). In metaphase II and in early telophase II the Golgi spheroids are conspicuous by their absence, but in late telophase II (fig. 2, B) the Golgi elements are once more (a third time) arising from the mitochondrial granules.

Spermateleosis and ripe sperm

Nath and Chopra (1955) state that in *Anadenus* the Golgi dictyosomes shift to the anterior aspect of the spermatid nucleus, where they generally arrange themselves in the form of an open U with its concavity facing towards the nuclear membrane. The chromophobic sphere in association with the U-shaped acroblast looks at this stage very dense, on account of the presence of a very large number of small acrosomal granules in it: these have been secreted by the acroblast (ref. text-fig. D, 1 in Nath and Chopra, 1955). Soon a large number of these tiny acrosomal granules are deposited just in front of the nuclear membrane in the form of a crescent-shaped cap (text-fig. D, 2 in the paper just quoted). Nath and Chopra have shown further that, a little later the acrosomal granules fuse to form a deeply-staining acrosomal cap, and the acroblast shifts back behind the nucleus to be sloughed off subsequently (text-fig. D, 3 in the same paper). Later on, the acrosomal cap is condensed into a small triangle, which, in the ripe sperm, forms a short spiral filament—the acrosome.

We have been able to photomicrograph all the stages of spermateleosis including the ripe sperm under phase-contrast in *Anadenus* and *Euaustenia*. In fig. 2, C, D, and E the U-shaped acroblast clearly betrays its origin from three

FIG. 3 (plate). Photomicrographs of living male germ cells of *Euaustenia cassida* under positive phase-contrast.

A and B, early primary spermatocytes, showing the crenated rods.

C and D, primary spermatocytes, showing various forms of Golgi bodies.

E, telophase I, showing some mitochondrial granules with higher phase-change.

F and G, secondary spermatocytes showing Golgi spheroids.

H, metaphase II.

I, spermatids (syncytium). The acrosomal granules are forming a cap over the nucleus.

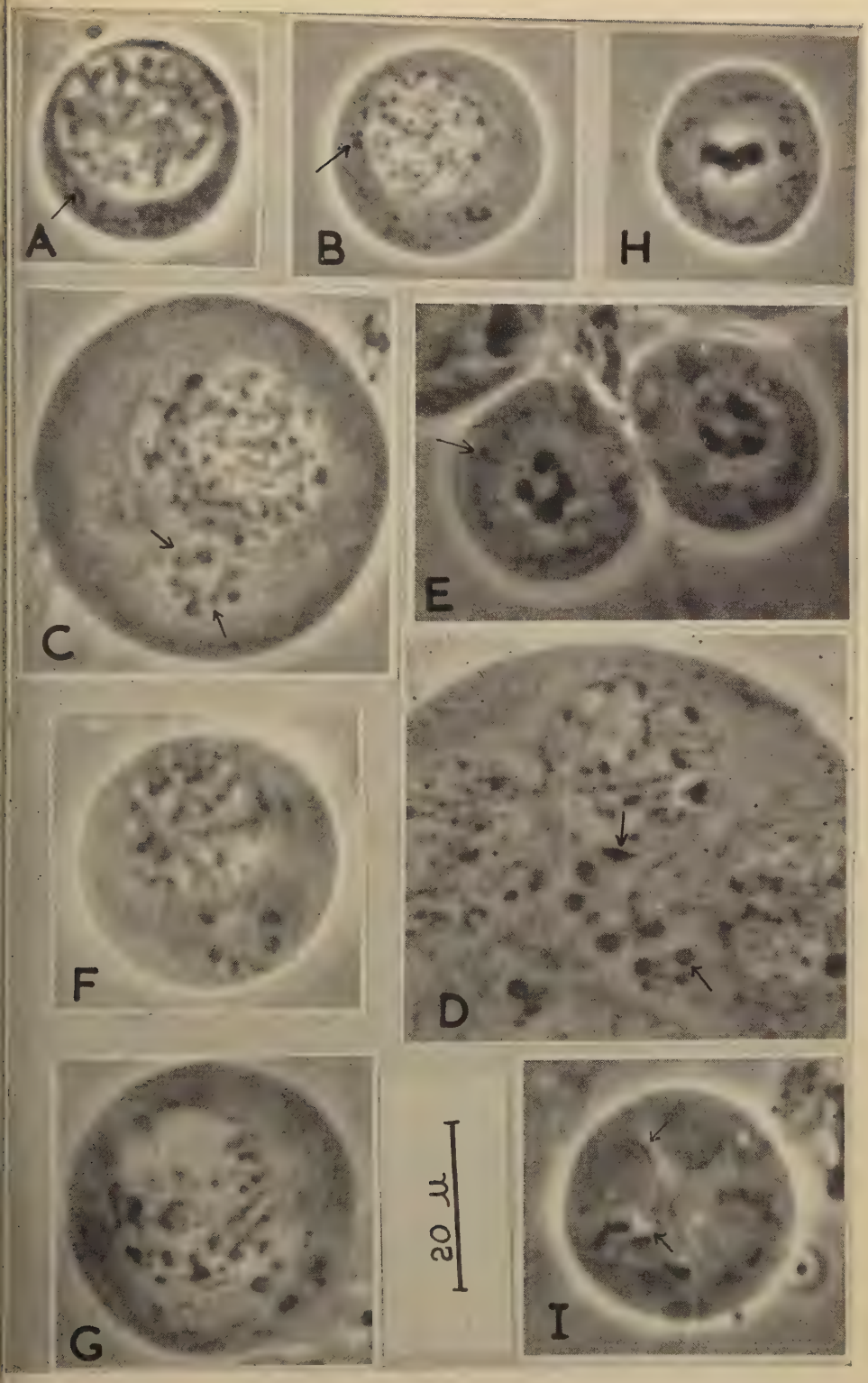

FIG. 3

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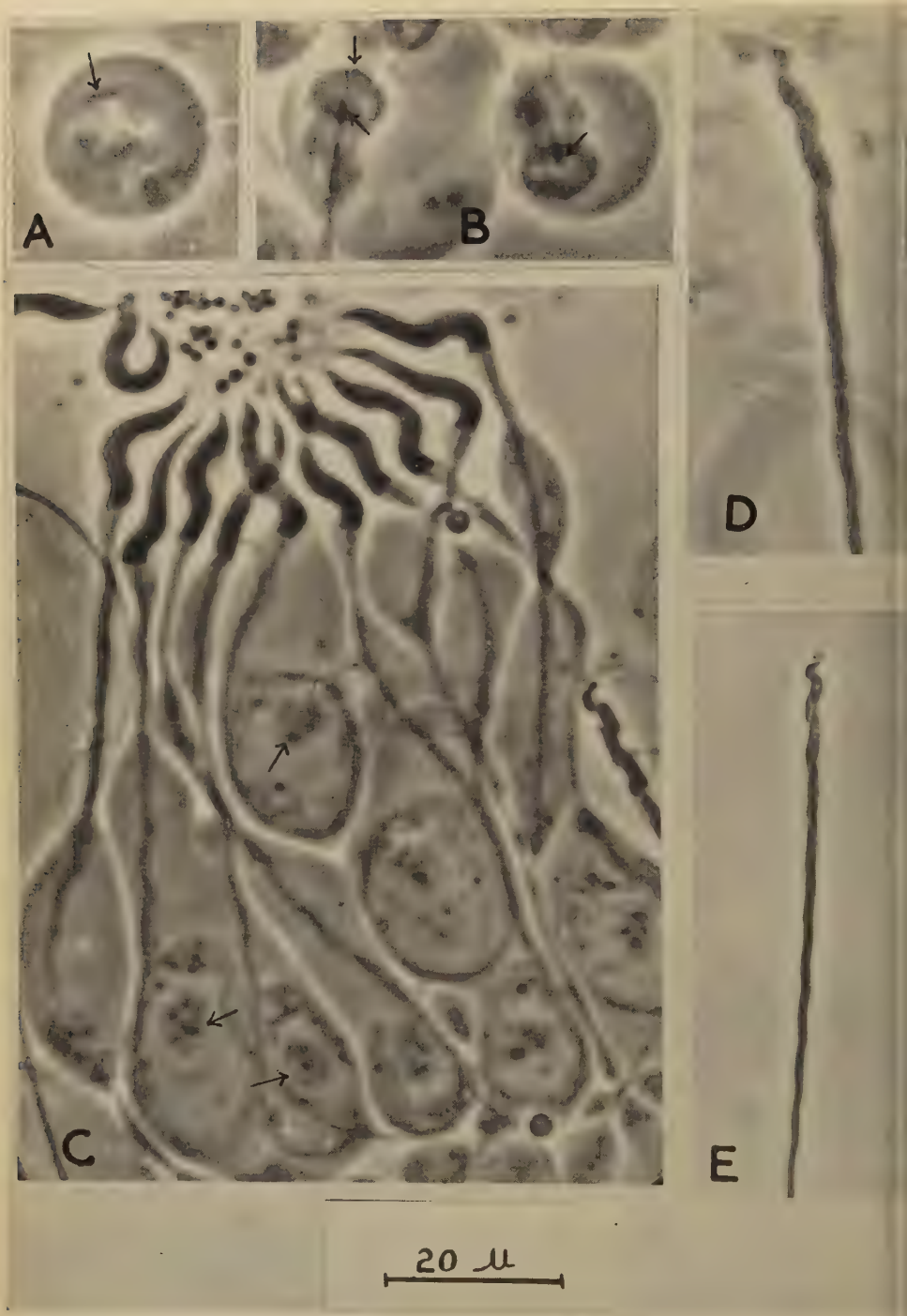

Fig. 4

V. NATH *and* B. L. GUPTA

Golgi dictyosomes. In fig. 2, C the sphere enclosed in the concavity of the U is very clear; in fig. 2, D some material is just flowing out of the concavity of the acroblast; and in fig. 2, E the thin cap of acrosomal granules in front of the nucleus appears in the form of a prominent bright streak, which makes an angle of about 135° with the bright material held up in the concavity of the acroblast. This last figure bears a very close resemblance with text-fig. D, 2 of Nath and Chopra (1955). The bright appearance of the cap of acrosomal granules is due to the fact that it is out of focus. This granular acrosomal cap is well in focus in fig. 3, I and appears dark, but the Golgi remnant is out of focus and appears bright.

The acroblast, having performed its function of acrosome secretion, shifts behind the nucleus (fig. 3, I), and the acrosomal cap sits down on the nuclear membrane (figs. 2, F and 4, A, B). The nucleus becomes dome-shaped, and at the same time the proximal centriole assumes the appearance of a rod (fig. 2, G and 4, B).

In the subsequent stages of spermateleosis the Golgi remnant degenerates and breaks up into a number of small spheres of greyish contrast, to be sloughed off along with the residual cytoplasm (fig. 4, C).

In the final stages of maturation the very long mitochondrial middle-piece, the much shorter nucleus, and the tiny filamentous acrosome all assume a spiral appearance (figs. 2, H–K and 4, D, E).

Staining with neutral red chloride

For supra-vital staining with neutral red chloride (B.D.H.), 0·01% of the dye was dissolved in the physiological solution used (see introduction). Observations made normally with bright-field illumination were checked under positive phase-contrast.

When the tissue is mounted in a drop of the physiological solution to which 0·01% of the dye has been added, the spheres of the Golgi spheroids begin to stain bright red in 20 to 30 minutes, as seen with bright-field illumination (fig. 1, H). By changing over to positive phase-contrast it is seen that neither the cortices of the spheres nor the rods and the granules in the Golgi region take up any stain whatsoever. About 1 hour after mounting, however, many unrelated structures in the cytoplasm of the primary spermatocyte begin to stain with neutral red, indicating that post-mortem changes have set in.

Discussion

Morphology of the Golgi spheroids

Roque (1954) has given a very detailed account of his observations on the living cells of the ovotestis of *Helix* as examined by phase-contrast and inter-

Fig. 4 (plate). Photomicrographs of living male germ cells of *Fuaustenia cassida* under positive phase-contrast.

A, spermatid, showing the dark acrosomal cap formed by the fusion of the acrosomal granules.

B to D, maturing spermatids.

E, mature spermatozoon.

ference microscopy. In late primary spermatocytes examined by phase-contrast, there are 'several (8–12) rods or crescent-shaped bodies', which are the 'most prominent objects in the cytoplasm because of their dark contrast'. But when 'these rods are studied in optical section by focusing at different planes it is observed that many of them are not rods but parts of composite bodies'. A composite body is described by Roque as 'a sphere partially or completely surrounded by a sheath of very dark contrast'. Some of these spheres are 'clear, vacuole-like; others look like solid bodies'. In addition to these composite bodies Roque describes 'granules and pale grey spheres of homogeneous structure'. But Roque also describes 'rods which do not appear associated with spheres: their rod-like appearance is not changed when examined at different planes'. Lastly, according to Roque, 'in many spermatocytes small granules are also observed in the cytoplasm adjacent to the paranuclear region'.

We entirely agree with Roque, except that in *Anadenus* composite bodies with complete sheaths, which would appear as rings in optical sections, are rather rare as compared with *Helix* and *Euaustenia*. This difference, however, is easily explained on the basis of variation.

Roque states that the composite bodies are the most numerous of these structures; and the granules and homogeneous bodies are not observed in every spermatocyte and their number varies from one cell to another.

When white light is used for observations by interference microscopy, the phase differences produced by parts of varying refractive index are transformed into colour differences. 'For example, on a yellow background of the cytoplasm, the composite bodies appear as violet-red spheres with a bluish-violet sheath. The rods also appear bluish-violet, and the granules and homogeneous bodies violet-red. The colours show that the sheaths of the composite bodies and the rods have a higher refractive index than the spheres and granules' (Roque).

We have not had the advantage of studying our material with the interference microscope, which, with white light, is capable of translating even very minute phase differences into different colours. In agreement with Roque we find that in *Anadenus* and *Euaustenia* the sheaths of the composite bodies and the rods have a very dark contrast as compared with the pale contrast of the spheres; but we are convinced that the granules in the Golgi region (paranuclear region of Roque) as well as the 'small granules' of Roque adjacent to this region have also a contrast as dark as the sheaths of the composite bodies and the rods.

Origin of the Golgi spheroids

Roque (1954) quotes La Valette St. George (1874), Prenant (1888), Benda (1898), Fauré-Fremiet (1910), Karpova (1925), Parat (1928), and Gatenby (1917, 1918), all of whom derived the dictyosomes (Golgi bodies) from the mitochondria.

Roque (1954) is himself doubtful about the origin of the Golgi dictyosomes (his paranuclear bodies), but the following paragraph is significant: 'Whether

the paranuclear bodies originate from mitochondria cannot be decided from this study, but the fact that in early primary spermatocytes a few paranuclear rods are surrounded by mitochondrial granules would suggest a common origin. The granules of the paranuclear region may represent transitional forms between the mitochondria and paranuclear bodies.'

Nath (1955) and Nath and Chopra (1955), working exclusively with Flemming-without-acetic and 0·5% iron haematoxylin, have shown (1) that it is in the earliest primary spermatocytes that the Golgi dictyosomes arise for the first time in spermatogenesis by the alignment of mitochondrial granules; (2) that during metaphase I there is no trace of dictyosomes left; (3) that during late telophase I the dictyosomes are again formed by the alignment of mitochondrial granules; and (4) during metaphase II and anaphase II the dictyosomes again break up to merge into the mitochondrial granules, to reappear again during late telophase II (for the third time in the course of spermatogenesis) by the alignment of mitochondrial granules.

Figs. 24 and 29 in plate 32 of Gatenby (1917), representing secondary spermatogonia of *Helix*, with crenated 'Nebenkern' (dictyosomes), bear a very close resemblance to the figures of Nath and Chopra (1955) in *Anadenus*, also drawn from Flemming-without-acetic and iron haematoxylin material, and also to the photomicrographs published in the present paper.

Watts (1952) in *Arion subfuscus* shows crenated dictyosomes in her text-fig. 5 (2), suggesting that the dictyosomes are formed by the alignment of mitochondria, although she does not offer any views in the text with regard to their origin.

In support of their claim that the Golgi dictyosomes are formed three times in the course of spermatogenesis in the slug, *Anadenus*, by alignment from the mitochondrial granules, Nath and Chopra (1955) quote Hirsch (1939). According to Bourne (1951), Hirsch believes that the Golgi 'pre-substance' (i.e. the initial Golgi granule) is derived from the mitochondria and later the pre-substance develops into the 'Golgi system', which consists of an 'externum' formed directly from the pre-substance alone and an 'internum' formed by the pre-substance with the co-operation of the cytoplasm and the mitochondria, the product of excretion later arising in the internum. Nath and Chopra (1955) stress the point that 'stronger evidence than *Anadenus* in favour of Hirsch's view could not be wished for, as the Golgi dictyosomes in the spermatogenesis of *Anadenus* can be shown not only to arise from the mitochondria but also to merge into them during metaphases I and II'.

It has already been mentioned that Roque (1954) does not rule out the possibility of the origin of his paranuclear bodies from the mitochondria, but he is doubtful about this origin. However, Roque (who studied living cells only) clearly states that (1) in spermatogonia and in very early spermatocytes paranuclear bodies are not observed and (2) the paranuclear bodies are not observed in dividing spermatocytes (of both meiotic divisions). These observations of Roque are in complete accord with the observations of Nath and Chopra (1955) in *Anadenus* and those of the authors of this paper in *Anadenus* and *Euaustenia*.

Secretion of the acrosome by the Golgi spheroids

Roque (1954), who did not prepare and examine 'fixed' permanent preparations of the ovotestis of *Helix*, failed to find any evidence 'of a direct participation of the paranuclear bodies in the formation of the acrosome'. The fact is that the process of acrosome-deposition is a very short-lived process in the animal kingdom in general and in the pulmonate gastropods in particular. It is, therefore, not surprising that, in the absence of permanent preparations, Roque failed to detect any connexion with the acrosome and his paranuclear bodies.

On the contrary, Nath and Chopra (1955) have given a very detailed account of the secretion of the acrosomal granules by the Golgi dictyosomes in *Anadenus*. At the very beginning of the process of spermateleosis the Golgi dictyosomes migrate to the anterior aspect of the spermatid nucleus. In this site the dictyosomes generally arrange themselves in the form of an open U with its concavity facing towards the nuclear membrane and enclosing a chromophobe sphere. Soon the dictyosomes (acroblasts) have deposited a cap of very fine granules, the acrosomal granules, in front of the nucleus, which can also be clearly seen in the chromophobe sphere (text-fig. D, 2 and pl. II, fig. 22 in the paper already quoted). The U-shaped structure of the Golgi complex is now changed into the familiar triangle formed by three dictyosomes holding the chromophobe material towards their inner surfaces. Soon after this the acrosomal granules fuse to form a deeply staining acrosomal cap; and the dictyosomes shift back behind the spermatid nucleus to be sloughed off in the late stages of spermateleosis. For further details of the differentiation of the acrosome and the nucleus in *Anadenus* reference may be made to the original paper of Nath and Chopra (1955) and to the section on Observations in this paper.

In our studies of the living ovotesticular material of *Anadenus* and *Euaustenia* with phase-contrast microscopy we have fully confirmed the details of acrosome-formation given by Nath and Chopra (1955), based on the study of material fixed in Flemming-without-acetic and stained with iron haematoxylin (figs. 2, C, D, E, and F; 3, I; 4, A, B, and C). Indeed, it will not be an exaggeration to say that it would have been very difficult, if not impossible, to work out the details in the living material but for the fact that one of us (V. N.) had thoroughly familiarized himself with the course of events in fixed *Anadenus* material. We wish particularly to refer again to our photomicrograph, fig. 2, E, in which the tiny acrosomal granules in the acrosomal cap have appeared in the form of a bright streak at an angle of about 135° with the granular material in the sphere of the U-shaped acroblast (see Observations). This photomicrograph seems to be an exact copy of text-fig. D, 2 and fig. 22 in pl. II published by Nath and Chopra (1955).

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Some Observations upon the Cytology of the Pars Distalis of the Surgically-removed Human Pituitary

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With two plates (figs. 1 and 2)

Summary

Human anterior pituitary tissue that had been removed at operation and immediately fixed was examined by a number of cytological and histochemical methods and by phase contrast and electron microscopy, and compared with similar material obtained *post mortem*. The general histological picture of good post-mortem material (not more than 4 hours *post mortem*) compared well with the surgically-removed tissue. For the study of silver impregnations of the Golgi substance, however, material removed at operation was found to be greatly superior.

Evidence was obtained showing that the intracellular lipid inclusions seen *post mortem* were not artifacts resulting from cytolytic changes. There appeared to be no relationship between these lipid bodies and the Golgi material as revealed by the Aoyama method.

No unequivocal dimorphism of the Golgi bodies, correlated with α- and β-cells, such as has been reported to occur in certain other mammals, was observed.

Phospholipid was present in the granules of a substantial proportion of the α-cells.

It was found that most of the cells which had been designated as β-cells after the application of certain routine staining methods, and most of the Gram-positive cells, reacted positively to the Periodic acid Schiff test: these cells could therefore be regarded as true β- or mucoid cells.

A method for the demonstration in frozen sections of the cell-types, together with the lipid inclusions, is described.

Introduction

SINCE so much has been written about the microscopical structure of the human anterior pituitary, some explanation is, perhaps, necessary for adding to the literature on this subject. The author believes, however, that since the bulk of the information available has been obtained from a study of post-mortem material, some useful purpose is served in describing certain aspects of the cytology of pituitaries rapidly and variously fixed in the operating theatre.

The material used can be described as normal in the sense that any structural changes due to surgical trauma, or the effects of anaesthesia, are probably less than those caused by a delay of several hours after death before fixation. On the other hand, since the specimens were obtained from patients with mammary carcinoma, it is not unlikely that there may be differences of another kind even in the absence of metastases—differences in the proportions of cell-types for example. This latter possibility was not investigated and attention was entirely devoted to aspects of the structure and staining properties of the individual cells, most of which could be regarded as normal.

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Materials and Methods

Ten pituitaries from women with breast carcinoma were available for study. The age range was from 41 to 52 years and in two instances there was evidence of carcinomatous infiltration into the pars distalis. In every case, small pieces of the gland were fixed immediately after removal. For comparison, three normal pituitaries obtained from 2 to 5 hours *post mortem* were used. The fixatives used, the reasons for their use, and the mode of embedding are listed below:

1. 10% neutral formalin in 1% $CaCl_2$ followed by paraffin embedding, for routine study. This fixative will be referred to as F/Ca.
2. Helly's fluid followed by paraffin embedding for general cytological study.
3. Fixation by method 1, followed by embedding in gelatine for the investigation of lipids and study by phase-contrast microscopy.
4. Aoyama's fixative followed by silver impregnation and embedding in gelatine for the investigation of the Golgi element.
5. Fixation in 1% osmium tetroxide buffered to pH 7·4 for electron microscopy.

Paraffin sections were cut at 5μ and were stained by:

a. The Periodic acid Schiff (PAS) technique according to Pearse (1949), with orange G as a counterstain.
b. The azocarmine technique as described by Friedgood and Dawson (1938).
c. Gram's stain as described by Foster and Wilson (1952).
d. Mallory's stain.

Of these, the first may be regarded as the most reliable for the demonstration of β-cells in the widest sense. The others were used as supplementary methods and their purpose is mentioned later.

The material embedded in gelatine (3 and 4 above) was cut as near 2μ as possible upon the freezing microtome and the sections were subsequently treated as follows:

a, with a filtered saturated solution of Sudan black in 70% alcohol or a similar solution of acetylated Sudan black for the demonstration of lipids, with subsequent mounting in Farrants' medium;
b, with Nile blue according to Cain's method (1947) for the demonstration of lipids;
c, with Baker's acid haematein test. Material from the same source, fixed in weak Bouin's fluid and extracted in pyridine was used as a control (Baker, 1946);
d, by a specially devised Ehrlich's haematoxylin/PAS/metachrome yellow technique as follows:
   1. After washing in water, treat with PAS as in Pearse's method.
   2. Wash thoroughly in running water and transfer to distilled water.

3. Ehrlich's haematoxylin for 5 minutes.
4. Blue in tap water.
5. Transfer to a 0·5% solution of Gurr's metachrome yellow in 30% ethanol and leave for 10 minutes or longer.
6. Rinse well in distilled water and examine under the microscope, when the α-cells should be yellow and chromophobes pale yellow to almost colourless. Further differentiation can be achieved by careful rinsing in 50% ethanol. The β-(mucoid) cells are reddish violet.
7. Mount in Farrants' medium:

This method was devised to afford a means of demonstrating the cell-types in frozen sections which were subsequently to be mounted in an aqueous medium (Farrants' medium). Acid dyes like acid fuchsin and orange G, commonly used for demonstrating the acidophils, were unsuitable owing to their solubility in water, but the water-insoluble dye metachrome yellow was found to be a satisfactory substitute. This method could be combined with the use of Sudan black and also worked successfully upon the gold-toned Aoyama material:

e, with hot methanol and chloroform in equal parts for up to 20 hours, to observe the effect of lipid extraction upon the PAS reaction;
f. by an acetylation technique (acetic anhydride in pyridine (Pearse 1953)). This was to observe the effect of acetylation upon the PAS reaction;
g, with Schiff's reagent without previous treatment, and after a preliminary treatment with cold 0·1 N. HCl. These procedures were to test for the presence of free or acetal aldehydes.

The thinnest sections of unstained gelatine-embedded material were also examined by phase-contrast microscopy, as were thin unstained paraffin sections of Helly material.

Finally, as some attention was given to a comparison of the results obtained from the use of different methods for the demonstration of β-cells, the procedure used is described below.

Paraffin sections 5μ thick of material fixed in neutral formalin were first stained by the azocarmine/orange G/aniline blue method (Friedgood and Dawson, 1938). A series of small groups of cells chosen at random were drawn with the camera lucida and careful notes of their staining characteristics were made. The dyes were then removed with a weak solution of Na_2CO_3 in 70% alcohol, and the section stained by the Gram technique. The same groups were then re-examined, and the Gram-positive cells among them noted. Lastly, the Gram complex having been destroyed by a short immersion in 70% alcohol, the section was treated by the PAS/orange G technique. On examining the cell groups again, the staining reactions of the cells were once more noted. A similar method was employed for studying the correspondence between Gram and PAS staining, after formalin fixation; and between azocarmine/orange G/aniline blue and PAS staining after Helly fixation.

Results

The general cytology and staining properties of the cells

Generally speaking, the fixation of the pieces of tissue was good. In some of the material embedded in gelatine there appeared, at first sight, to have been some degree of shrinkage. Any distortion, however, was located in the embedding medium filling the intercellular spaces; the cells were unaffected.

After fixation in either F/Ca or Helly's fluid, embedding in paraffin, and staining with the azocarmine method of Friedgood and Dawson, the cells could be fairly easily classified according to the staining properties of their granules. On such a colour basis, it was found that the cells could be divided into the following groups: (*a*) red to orange, (*b*) orange-purple, (*c*) purple, and (*d*) blue. The chromophobes formed a separate class and the coloration of their cytoplasm was pale and somewhat variable. Longer differentiation tended to reduce the intensity of the blue staining and, if carried to extreme, would presumably have eliminated this class of cells altogether. Similarly, the affinity of the cells for azocarmine as compared with orange G, appeared to depend upon the times of staining and differentiation. In consequence, it is not proposed to suggest any correspondence between the cells taking up azocarmine in man and the carminophils described by Friedgood and Dawson in the rabbit (1938).

In previous studies based on human post-mortem material (Foster and Wilson, 1952), an investigation was made into the anterior pituitary cells whose granules reacted positively with a Gram technique. It was concluded that the Gram-positive cells were always β-cells (or mucoid cells, according to the terminology of Pearse). When, in the present investigation, groups of cells fixed with formalin, embedded in paraffin and stained by the azocarmine method were decolorized, subjected to the Gram technique, and re-examined, it was found that all cells coloured red or orange were Gram-negative. Further, all cells whose granules were coloured orange-purple or purple by the first method were Gram-positive by the second. Cells, however, whose granules were stained blue might be either Gram-positive or Gram-negative. No differences between these two classes of cell could be determined. If now the Gram staining was removed and the same fields examined after treatment by the PAS/orange G technique, it was found that the granules which had been coloured red or orange by the azocarmine method were stained orange by the latter and were thus PAS negative, whereas all cells whose granules were coloured orange-purple, purple, or blue by the former were invariably Schiff-positive by the latter. Since it is likely that all PAS-positive cells are β-cells of one kind or another (i.e. thyrotrophs or gonadotrophs—if such exist separately in the human pituitary) it may be stated that, with the particular trichrome technique used, all granulated cells which stain unequivocally red or orange are α-cells and the rest are β- or mucoid cells. This statement applies also to material fixed in Helly's fluid.

From what has just been said, and from the further study of material fixed with formaldehyde and treated by the Gram and PAS techniques only, it was concluded that a high proportion of PAS-positive cells were also Gram-positive, but that Gram-positive cells reacting negatively to the PAS method probably never occur.

Some consideration was given to the relative sizes of the granules in the cells mentioned above. The observations were made on fixed material, since living material was not available.

The most detailed account of the granule characteristics of human anterior pituitary cells is undoubtedly that of Romeis (1940). He described a range of granule size in individual cells of each type of chromophil, the greatest range being shown by the α-cells. In the present material, although there was variability in size, it could not, in general, be considered to be very great either within any individual cell or as between cells of the same class. There can be little doubt, however, as Romeis has pointed out, that the type of fixative used may have a marked effect upon the size of the granules. In this material it was noticed that after Helly fixation there was, for the most part, no significant difference between the size of the granules of α- and β-cells, but after formalin fixation the α-granules appeared decidedly coarser, possibly owing to clumping. Both after Helly and formalin fixation, there was evidence of a numerically small class of β-cells in which the granules were larger than the normal. After the former fixative they coloured either blue or purple with Mallory's stain and, after the latter fixative, they coloured blue with the Azan method. Material fixed with formalin and embedded in gelatine revealed no evident size differences between cell granules when examined by the phase-contrast microscope.

In sections of material fixed in Helly, embedded in paraffin, and stained by the Azan method, and also in sections of material fixed in formalin, cut on the freezing microtome, and treated by the PAS technique, cells were observed which, because of their definitive staining, clearly belonged to the mucoid or β-type. These showed, however, no sharply resolvable granules either with the direct light microscope, with a variety of filters, or by the phase-contrast microscope.

In several instances, masses of nucleated cytoplasm were observed in which no cell boundaries were discernible—the arrangement being much more like that of a syncytium. The granulation was quite marked and, after the azan technique, the granules of some masses coloured red and others a dull purplish blue. The former, with the PAS/orange G technique were orange and the latter violet. These apparent syncytia thus conformed in their staining reactions to the normal α- and β-cells.

Finally it was evident that the small chromophobes lacked specific granules although the cytoplasm did in fact appear granulated. This was noticeable also when unstained thin frozen sections were examined with the phase-contrast microscope. Such granulation was, presumably, partly mitochondrial and partly due to flocculation of the cytoplasm resulting from fixation. The

staining of such cells was variable and never as intense as that of the chromophils.

The lipid inclusions

As can be seen from the figures of Romeis (1940) and fig. 2, C, the cells of the human pars distalis were exceedingly rich in lipid inclusions. Since they were as abundant in surgically removed as in post-mortem material, it is evident that they do not represent a degeneration phenomenon. Inclusions of comparable size and frequency have not been observed in the rat (Foster, 1947), the rabbit, or the rhesus monkey (Foster, 1955). In spite of their obviousness after appropriate techniques it appears that little is known about their function.

A study of the lipid inclusions in F/Ca frozen sections coloured with Sudan black confirmed the careful observations of Romeis and other earlier workers. There was a considerable range both in size and form, from small apparently solid granular bodies to vacuolated structures as large as the nucleus. In the latter the lipid was associated with the surface of the empty-looking vacuole. In the smaller vacuolated inclusions the lipid part commonly formed a thick, completely enveloping rind. In the largest the lipid occurred as a uniform continuous thin film, a crescentic layer, or a series of separate, flattened lipid particles, perhaps joined by an exceedingly thin lipid layer (fig. 2, A and D). The larger vacuolated lipid bodies were readily deformed in the cells by, for example, undue pressure on the coverglass. Thin, uncoloured sections mounted in Farrants' medium and examined by the phase-contrast microscope showed clear vacuoles, sometimes with a darker rim and sometimes without. On colouring with Sudan black and re-examining the same cells, the vacuoles were observed to have sudanophil material at the periphery (see fig. 1, A, B, D, E). Under phase contrast the smaller, virtually solid bodies were not readily separable from the granulations of the cytoplasm.

In addition to the simple forms just described, there were multiple, vacuolated, mulberry-like bodies with a sudanophil surface layer (figs. 1, A and 2, A). These were generally rather large. Electron microscopy of ultrathin sections of surgically-removed and post-mortem material fixed in osmium tetroxide suggested, however, that even some of the smaller inclusions may contain a multiple vacuolar system within the lipoid cortex (fig. 3, B). At the same time, this technique also demonstrated that many of the smaller inclusions were probably solid.

FIG. 1 (plate). These are all thin frozen sections of surgically removed tissue, fixed in F/Ca and embedded in gelatine.

A, mainly mucoid cells showing large vacuoles with lipid at their surfaces. Coloured with Sudan black.

B, the same field as above. Unstained, phase contrast.

C, low-power view of section coloured with Sudan black to show the general distribution of the lipid inclusions.

D, these are principally α-cells coloured with Sudan black. *l*, α-cell with several smaller vacuolated inclusions with sudanophil rims.

E, the same field as D. Unstained, phase contrast.

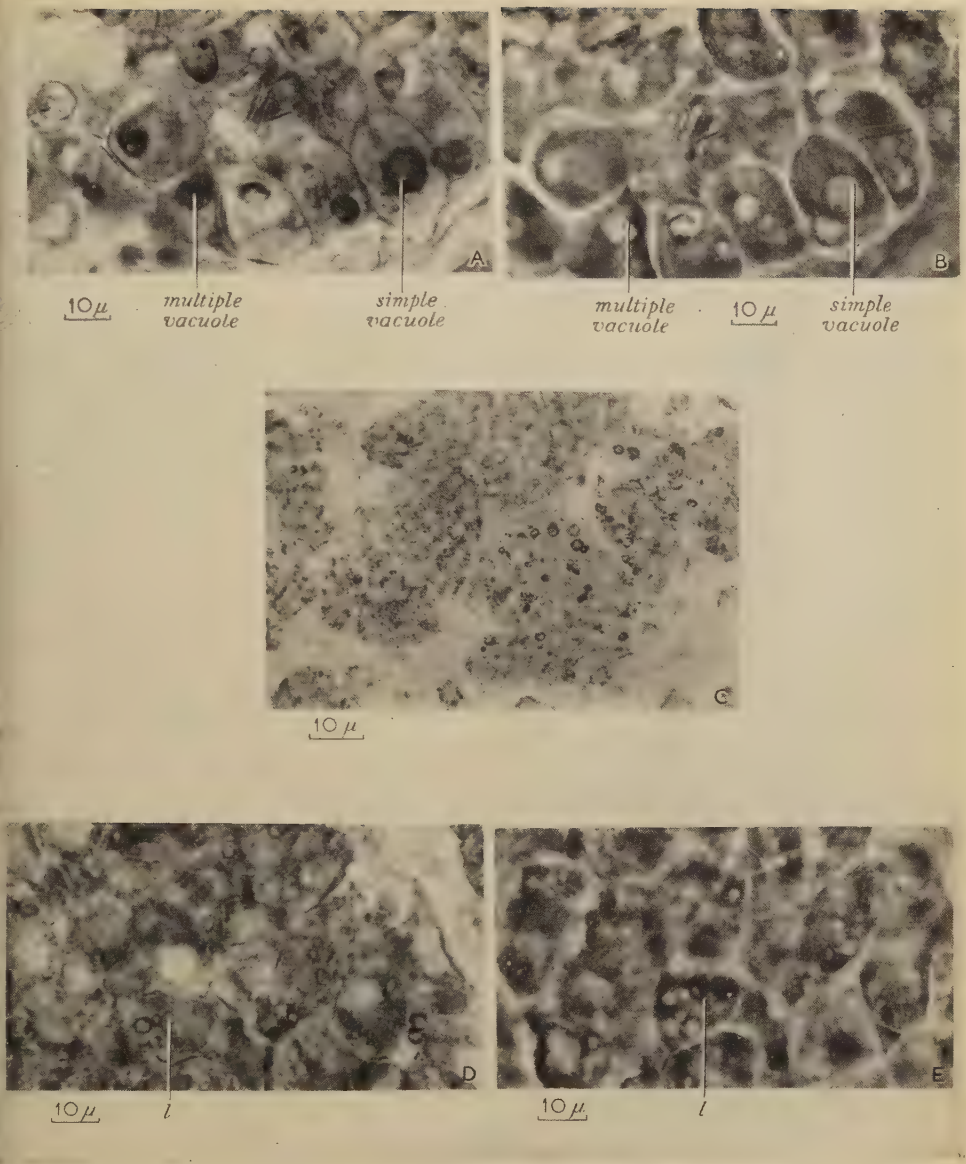

FIG. 1

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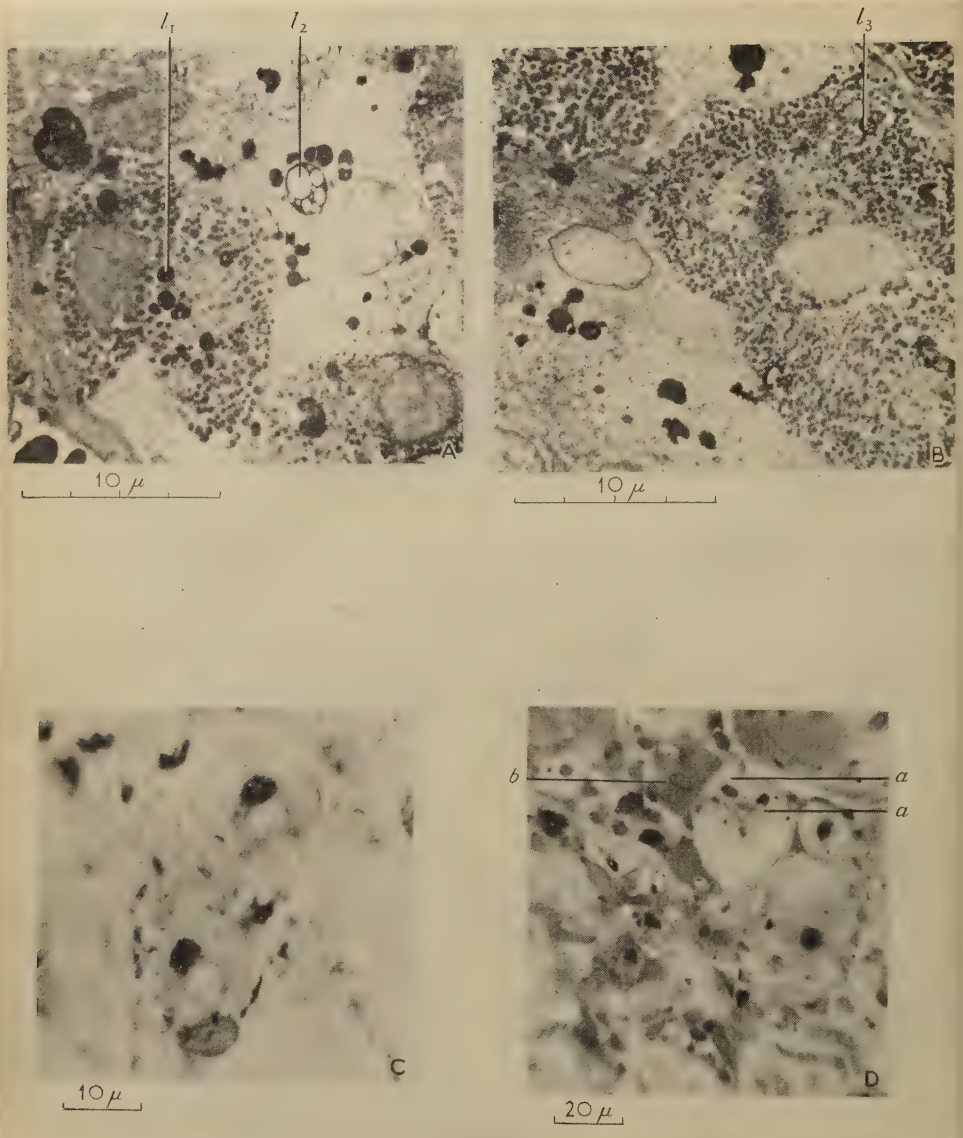

FIG. 2

C. L. FOSTER

The largest sudanophil vacuoles were predominantly associated with the mucoid cells and sometimes the vacuoles were larger than the nuclei (fig. 1, A). One or two such structures were observed to occur in a cell and they were often accompanied by a scattering of smaller sudanophil droplets, with or without vacuoles. The α-cells and the chromophobes contained smaller sudanophil vacuoles and particles. These were often very numerous (fig. 1, C).

After the acid haematein test, the lipid inclusions were coloured, but extremely faintly. This faint reaction, however, may indicate the presence of small quantities of phospholipid. In control material subjected to the pyridine extraction test the lipid bodies were completely uncoloured.

The most striking finding after using this test was observed in certain chromophil cells, in which there were large numbers of strongly reacting granules. Groups of such cells were drawn with the camera lucida and the sections then treated by the PAS/metachrome yellow method and re-examined. This procedure removed the acid haematein coloration, but produced a sufficiently strong staining in the mucoid cells to enable them to be easily identified. It was found that the cells positive to acid haematein were always PAS-negative. It is considered probable, therefore, that the cells concerned are α-cells and that the reacting granules are their specific granules. When sections treated by the technique were additionally stained with orange G, there appeared to be some α-cells whose granules had failed to blacken and which, therefore, coloured orange. In control sections of pyridine-extracted material, cells positive to acid haematein were not observed and it is concluded in consequence that phospholipid is a component of the granules of many, although perhaps not all, α-cells.

As has been shown by Pearse (1952*a*) and others, there are certain lipid bodies which are also strongly PAS-positive. These were observed predominantly in chromophobes and β-cells, but were also noted on occasion in α-cells. These, although by no means as frequent, resemble in many respects some of the sudanophil but PAS-negative bodies described above. None, however, comparable with the largest of the former have been observed. The PAS-positive material appeared to correspond very precisely in its distribution to that of the sudanophil substance, the central vacuole, where present, being PAS-negative.

It was not possible by staining techniques to demonstrate any solid material

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FIG. 2 (plate).

A and B are electron micrographs of ultra-thin sections of material between 2 and 3 hours *post mortem*. Fixed in 1% osmium tetroxide. l_1, medium-sized, perhaps solid lipid body. l_2, large multiple lipid inclusion. l_3, similar lipid body, but very much smaller; this may be an intrinsically smaller body, or it may represent a tangential section through one of the larger ones. The granulated cells shown in these photomicrographs are probably α-cells.

C, two α-cells with Golgi bodies from a thin frozen section. Aoyama, treated with gold chloride and stained by the PAS/metachrome yellow method.

D, cell *b* is a mucoid cell with a somewhat flattened Golgi element adjacent to the nucleus; *a*, α-cells. Each α-cell shows a Golgi network, but the nucleus is seen in only one of them. (Technique as in C.)

within the vacuoles, although with phase-contrast microscopy what appeared to be pale flocculated material could occasionally be seen.

With acetylated Sudan black (kindly supplied by Dr. Bruce Casselman) the lipid inclusions coloured, but not so strongly as with the normal form. There was, however, much less background coloration. When Nile blue sulphate was applied according to Cain's method the lipids coloured blue, but on further differentiation there was a slight change towards purple suggesting, perhaps, the presence of both acidic and neutral lipids.

Treatment of formalin-fixed frozen sections with boiling methanol and chloroform for 20 hours or more, completely removed the lipid, since subsequent treatment with Sudan black was without effect. This treatment, however, did not eliminate the PAS-positive reaction, either of the mucoid granules or of the PAS-positive lipid bodies, but the intensity of the reaction seemed to be slightly decreased. From these results it was concluded that the lipid part of the latter was fairly readily separable from the PAS-positive part.

When formalin-fixed frozen sections were acetylated with acetic anhydride in pyridine, the PAS reaction became negative both in the β-cell granules and in the lipid inclusions. De-acetylated sections, however, reacted in a precisely similar manner to untreated controls. It was thus presumed that the PAS-positive reaction of the lipid bodies, like that of the mucoid granules, was due to the presence of 1–2 glycol groupings.

There was no coloration by Schiff's reagent of untreated frozen sections or in those treated with hydrochloric acid. This suggests the absence of free or of acetal aldehyde.

The Golgi material

The post-mortem material proved unsatisfactory for the demonstration of clear-cut Golgi networks by the Aoyama method. In two of the surgically-removed specimens, however, good impregnations were obtained, quite comparable with the results to be expected after the rapid fixation of animal material.

It is not proposed in this paper to discuss the nature of the networks obtained by techniques of this sort. It is sufficient to point out that whatever the materials or structures are that reduce the silver nitrate—and they may differ in different types of cell—they are almost certainly functionally significant within that cell.

A dimorphism of the Golgi material with respect to the two main kinds of chromophil cell has been reported in the mouse (Urasov, quoted by Romeis, 1940), the cat (Atwell, 1932), and the rat (Severinghaus, 1937; Foster, 1947). Romeis (1940) has also described a similar condition in man. In all these instances the Golgi material of the α-cell has been described as a small rather compact network generally in close association with the nuclear membrane, whereas that of the β-cell is a larger structure consisting of loose threads arranged in a wider mesh and lying in a free state separated from the nucleus.

In this investigation thin frozen sections of Aoyama material embedded in

gelatine were used. After treatment with gold chloride, the mucoid cells were demonstrated by the PAS method and the α-cells by staining in metachrome yellow. An examination of a number of camera lucida drawings of α- and β-cells chosen at random showed that although the Golgi nets of β-cells appeared *generally* to be separate from the nuclear membrane (figs. 2, D, and 3, H, I), those of α-cells might be either in contact with or quite separate from it (figs. 2, C, D, and 3, E, G). In view of this, it was not possible to substantiate the findings of Romeis in this matter. In the small chromophobes, the Golgi material was somewhat variable in form, ranging from small, compact, juxtanuclear networks to more dispersed rather granular aggregations (fig. 3, A, B, D).

In many α- and β-cells the Golgi material took the form of a much more widely spread and often fragmented mesh of threads and granules (fig. 3, F, J, K, L, M, N), and in such instances, because of the similarity of form, there was even less evidence to suggest a clear-cut dimorphism of the Golgi material. It was often noticed that the dispersion of the Golgi material just described occurred in cells almost depleted or only partially filled with their specific granules. It is not unlikely that this particular form of the Golgi substance is associated with a process of granule-formation going on within such cells, since it is known that the configuration of the Golgi material changes with the functional state of the cells (Foster, 1942; Bourne, 1951).

No evidence was obtained for any relationship between either the PAS-positive or the normal lipid inclusions and the Golgi substance, since in the α-cells and small chromophobes the inclusions were widely scattered throughout the cytoplasm and, in the β-cells, the Golgi material and the large, vacuolated lipid bodies were commonly observed together in the same cell. It did not appear, therefore, that the Golgi element consisted of silver deposited upon the surface of the *demonstrable* lipid of the cell during the Aoyama technique. Furthermore, no diffuse lipid colourable with Sudan black was evident, as it was in the anterior lobe cells of the rat (Foster, 1947).

It is hoped to publish a separate account of the Golgi material of these cells.

Discussion

A general correspondence in the delineation of cell-types resulting from the use of the Gram and Azan methods on the one hand, and the PAS/orange G technique on the other, was observed. It is considered advisable, however, that all routine staining techniques to be used should first be checked against the PAS/orange G method.

A relatively small proportion of mucoid (β-) cells were Gram-negative, but no other differences were detectable. Whether these cells constitute a chemically valid, separate category is open to question, since in the rabbit pituitary, for example, the kind of cell which reacts positively to the Gram method depends very largely upon the fixative used. In the rabbit, after neutral formalin fixation, it is the α-cell which reacts positively. Nevertheless, these cells in man may correspond to the γ-cells of Pearse, but since there is little

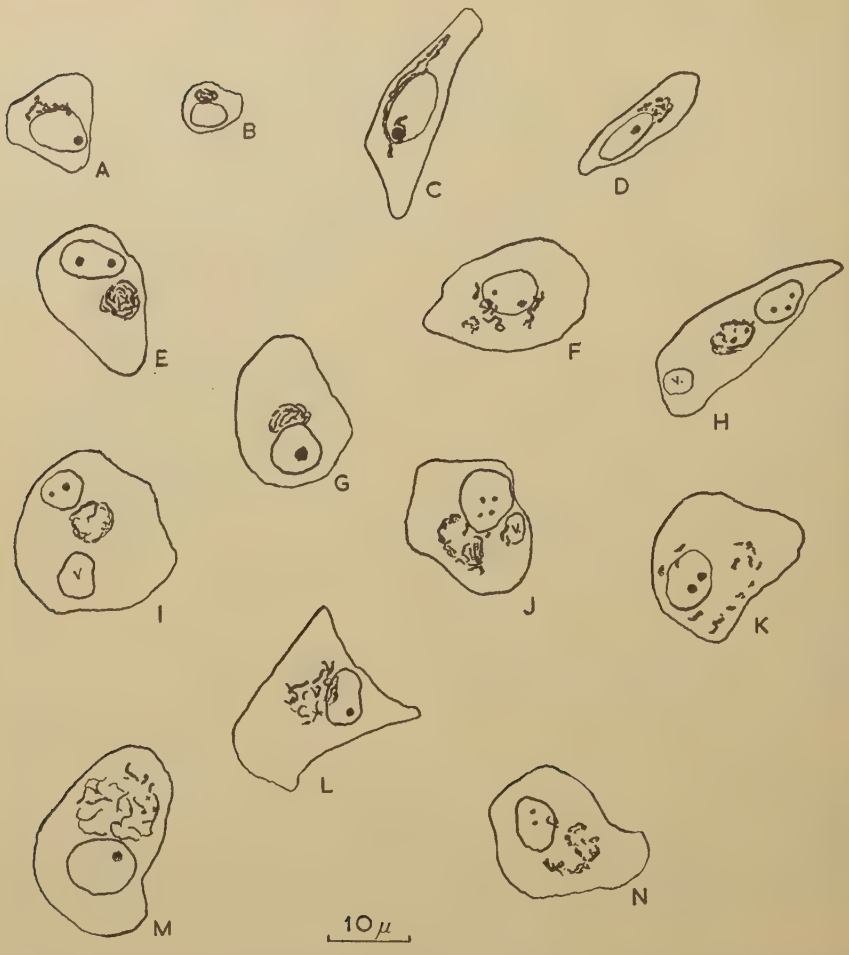

FIG. 3. Camera lucida drawings of cells from a surgically-removed pituitary. Aoyama technique, gold toned, PAS/metachrome yellow. 3 μ frozen section mounted in Farrants' medium. No specific granules are indicated in these drawings. A, small chromophobe with small, somewhat granular Golgi body. B, very small chromophobe with compact Golgi network. C, probably immature mucoid cell, with moderate number of PAS-positive granules. Golgi element somewhat resembling the small chromophobe type. D, small chromophobe with moderately compact Golgi body. E, mature, granule-filled α-cell with Golgi body separate from the nuclear membrane. F, mature α-cell with fragmented Golgi element and sparse granulation. G, mature granule-filled α-cell with Golgi body adjacent to the nuclear membrane. H, mature well-granulated mucoid (β-) cell with Golgi element free in the cytoplasm. V = lipid vacuole. I, similar to H. J, mature α-cell with sparse granulation and somewhat expanded Golgi body. K, partially granulated mucoid cell with very dispersed and fragmentary Golgi element. L, partially granulated mucoid cell, with expanded Golgi body in juxtaposition to the nuclear membrane. M, moderately granulated mucoid cell, but not strongly PAS-positive. Much expanded Golgi element with threads in association with mucoid granules. N, large chromophobe of mucoid origin with a few granules in the cytoplasm. The Golgi body rather expanded and granular.

evidence that the chromophobic cells so designated by Romeis (1940) correspond with them, an alternative term might be preferable.

According to Pearse (1952*a*), since a positive Gram reaction in their granules defines most, but not necessarily all, mucoid cells, it would be expected that in the same field successively stained by the Gram and PAS methods there would be found either more PAS-positive cells than Gram-positive ones or else equal numbers of each. In figs. 1 and 2 of Pearse (1952*a*), as far as can be judged from the photomicrographs, there appear to be slightly more Gram-positive than PAS-positive cells. If this is so, then presumably some α-cells are Gram-positive; such cells were never observed in the present investigation.

Certain PAS-positive cells, in which granules could not be resolved by any of the methods used, may correspond to the 'disperse' cells of Pearse, and those with significantly larger granules staining blue or purple after Helly fixation followed by Mallory and blue in the azan technique after formalin, may be equivalent to the 'punctate' cells of the same author (Pearse, 1952*b*).

The granulated syncytia appeared to correspond to those described by Romeis (1940). He described only eosinophil syncytia or 'plasmodia' as he called them, but in the present material mucoid syncytia were also observed from time to time.

The significance of the presence of phospholipid in the specific granules of a substantial proportion of the α-cells is obscure. If a similar condition obtained also in mammals more readily amenable to experimental procedures, then it might become possible to elucidate the functional significance, if any, of the phospholipid. In this connexion, two reports upon the presence of a positive acid haematein reaction in the α-cells of the rat pituitary have been noted in the literature (Rennels, 1951; Finerty, Hess, and Birnhammer, 1952). It was felt, however, that little reliance from a histochemical point of view could be placed on these observations. In the first place, Rennels departed from Baker's original technique by using paraffin sections, and secondly he reported a *cytoplasmic* reaction of a similar kind, both in those sections subjected to the acid haematein test, and those subjected to the pyridine extraction test. In view of this, no inferences relating to the presence of phospholipid can properly be drawn. Finerty and his colleagues, using the same procedure (which they referred to as Rennel's modification of the acid haematein test), also described a positive reaction, but this time in the *granules* of the α-cells of the same animal. Whether this result was also obtained in the pyridine-extracted controls was not stated.

The distribution and forms of the lipid particulates in the cells of post-mortem and surgically-removed material appeared to be identical. It was thought highly improbable, therefore, that the lipid bodies were indicative of degenerative changes. The association of one or two large lipid vacuoles with mature mucoid cells, and the association of more numerous smaller lipid bodies with mature α-cells did not seem to be so firmly established as the account of Romeis suggests. Intermediate conditions were not infrequently

observed. Neither did it appear that the PAS-positive lipid inclusions were absolutely restricted to mucoid and chromophobic cells, as Pearse indicated, since in occasional instances, such structures were observed in unequivocal α-cells. With regard to the chemical nature of the latter bodies, the conclusions reached are substantially in agreement with those of Pearse, to the effect that there is both lipid and mucoprotein, although it is not possible to say with certainty whether some of the lipid itself may not be PAS-positive. It was noticed, however, that extraction with fat solvents did appear to lessen somewhat the intensity of the PAS reaction. The significance of these two kinds of lipid inclusions must still remain a matter for speculation, but since the total amount of fatty material associated with them is very substantial they are presumably not without functional importance. As was mentioned earlier, such large lipid inclusions were not observed in the single example examined of the pituitary of the rhesus monkey, but it would be of interest to know whether they occurred in other members of the Anthropoidea.

The faint reaction of many of the lipid inclusions with the acid haematein test may indicate that a small fraction of their lipid is in the form of phospholipid. It was not possible to determine whether this observation applied both to PAS-positive and PAS-negative lipid.

No exact and invariable correspondence, topographical or otherwise, was observed between either type of sudanophil lipid body and the Golgi structures as revealed by the Aoyama technique. This was in sharp contrast to the condition in the rat, where the Golgi material as shown by osmium tetroxide impregnation corresponded very exactly with the diffuse and the minutely particulate sudanophil lipid (Foster, 1947). It is possible, however, that the Golgi substance in the human cells might be associated with a 'masked' lipoid which would not necessarily colour with Sudan black.

Again, in this material, unlike that of the rat (Severinghaus, 1937) and the cat (Atwell, 1932), no clear-cut dimorphism of the Golgi material in relation to the mature acidophil and the mucoid cell-types was observed, although such dimorphism is claimed for man by Romeis (1940). Pearse's results also do not support Romeis in this matter, although it appears doubtful whether any conclusions can be safely drawn about the Golgi substance of most post-mortem material. In the only pituitary in this series which was subjected to the Aoyama technique *post mortem* (less than 3 hours), the picture obtained was very much less satisfactory than that found in some of the surgically-removed glands. In many cells, and particularly in those not fully charged with granules, the Golgi material was fragmented and extensively dispersed through the cytoplasm. It seemed likely, by analogy with glandular cells of other kinds, that this condition was correlated with the physiological state of the cells concerned. More, however, must be learned about secretory cycles in anterior pituitary cells and about the ultra-structure of the Golgi area before any more definite statements can reasonably be made.

I would like to thank Dr. R. R. Wilson, formerly of the Department of

Morbid Anatomy, for his invaluable co-operation during the early stages of this investigation, particularly for the photomicrographs in fig. 1, and Dr. Cyril E. Challice, also of St. Mary's Hospital Medical School, for the preparation of the electron micrographs. Thanks are also due to Mr. E. J. Radley Smith of the Royal Free Hospital, London, and Mr. A. Dickson Wright of St. Mary's Hospital, London, for providing the material. Finally, I am indebted to Mr. H. Long, histology technician of the Department of Biology, St. Mary's Hospital Medical School, for much technical assistance.

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Some Cytological Characteristics of Thyroidal Function in the Endostyle of the Ammocoete Larva

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With two plates (figs. 2 and 3)

Summary

An account is given of the distribution of thyroidal function in the epithelium of the endostyle of the ammocoete larva, based on the localization of bound iodine as revealed by autoradiographs; and the cytological characteristics of the various types of cells are analysed in relation to this. The binding of iodine is shown to be correlated with the production of a PAS-positive 'thyroidal prosecretion' which, in fully active cells, tends to become transformed into a more granule-like phase. These thyroidal granules appear to have some degree of permanence and to enlarge by the accumulation of material; the precise significance of this is not clear, but it might involve the laying-down either of secretory reserves or of the by-products of thyroidal metabolism. The granules differ from the initial phase of the prosecretion in developing a brown pigmentation, a positive ferric-ferricyanide response, and a yellowish fluorescence, together with some affinity for orange G. These properties, together with the PAS-positive response of both phases of the prosecretion, give considerable support to the view that the secretory process involved is homologous with that which produces thyroid colloid in higher forms, although the histochemical reactions concerned are not sufficiently specific to be decisive. Thyroidal function is widely spread in the epithelium, except in the glandular tracts (type 1 cells), but it varies in its degree of expression; the pattern of this, and its relationship to the alimentary activity of the endostyle, can conveniently be described in terms of the embryological concept of 'gradient fields'. The present results are discussed in relation to the fate of certain regions of the epithelium at metamorphosis.

Introduction

THE occurrence of thyroidal biosynthesis in the endostyle of the ammocoete is now well established (Gorbman and Creaser, 1942; Leloup and Berg, 1954), but its cytological basis is little understood; moreover, it has been stated that those parts of the endostylar epithelium which are thought to play a major part in this thyroidal activity make only a minor contribution to the gland of the adult (Marine, 1913; Leach, 1939; Sterba, 1953), so that the actual relationship of the latter to the larval organ is obscure. In order to clarify this situation we have therefore carried out a renewed and more detailed study of the distribution of thyroidal function in the endostyle, and have attempted a correlation of this with the cytological characteristics of the epithelial cells. The present account deals with the conditions in the normal animal, and we hope to report later on the results of experimental treatment.

Material and Methods

The animals used were older larvae (60 to 145 mm), collected in Derbyshire and Hampshire, and probably all belonging to the species *Lampetra*

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planeri. For general study the pharynx was fixed in mercuric-formaldehyde and stained by the Azan technique; mucicarmine and toluidine blue were used for the identification of mucin, while mitochondria were stained by the Kull technique, after fixation in Helly's fluid with post-chroming, or in Champy's fluid. Histochemical tests, selected with special reference to the known properties of thyroid colloid, included the hydrogen peroxide, Nile blue sulphate, and Fontana tests for cell pigments (Pearse, 1954), the Periodic acid Schiff (PAS) procedure, the ferric-ferricyanide test (Lillie and Burtner, 1953; Pearse, 1954), and the methyl green pyronin procedure for RNA (with ribonuclease or perchloric acid as extraction agents for control preparations), together with the use of fluorescence-microscopy. Autoradiographs were prepared with Kodak stripping film, according to the procedure of Pelc (Pearse, 1954), the animals having previously been immersed for 48 hours in water containing 200μc of ^{131}I per litre; before this most had been in clear water for one or more weeks, but some were transferred direct to the radio-iodine solution from mud. There was considerable variation in the intensity of these autoradiographs, and further investigation will be needed to determine how far this is influenced by the age and provenance of the animals; it does not, however, affect our general propositions.

Observations

General organization of the endostyle.

Detailed accounts of this will be found in the works of Marine (1913), Kieckebusch (1928), Leach (1939), and Sterba (1953). Despite the morphological complexity of the organ, its cytological characteristics remain similar in principle throughout, and the present account will therefore be based for simplicity on conditions in the anterior region. The two chambers of this are mirror-images of each other, and are lined (fig. 1) by an epithelium which is, in origin, an extension of the alimentary tract, being continuous with the pharyngeal epithelium at the vertical canal by which the endostylar lumen is in communication with that of the pharynx.

Previous authors have attached importance to the differentiation of the endostylar epithelium into a number of zones, and these will be referred to here in terms of the cell 'types' enumerated by Marine (1913) and Leach (1939); there is, however, as we shall show, a marked tendency for the actual functioning of the latter to be organized along gradients of differentiation rather than by the sharp demarcation of regions.

Type 3 cells. It will be convenient to begin with these cells (fig. 1), since they are known to be active in the binding of iodine (Gorbman and Creaser, 1942), and it is in them, in particular, that the cytological characteristics of thyroidal function must therefore be sought. They are broad, columnar cells, with a dense covering of short cilia, which, according to Kieckebusch (1928), are non-motile, although this has been questioned by Sterba (1953). Over most of the type 3 area the nuclei are vesicular, each with a prominent nucleolus which is not, however, so large as those of the type 1 cells; the staining reaction

of these nuclei varies from acidophil to basiphil, as noted by Sterba. RNA is conspicuous, but is sharply polarized, being concentrated below the nucleus in a dense and compact zone of cytoplasm (figs. 2, F; 3, A; 4, B (see p. 398)). Thread-like mitochondria are present basally, but are not always clearly resolvable and are usually much more slender than those found in certain other regions of the epithelium (see p. 401).

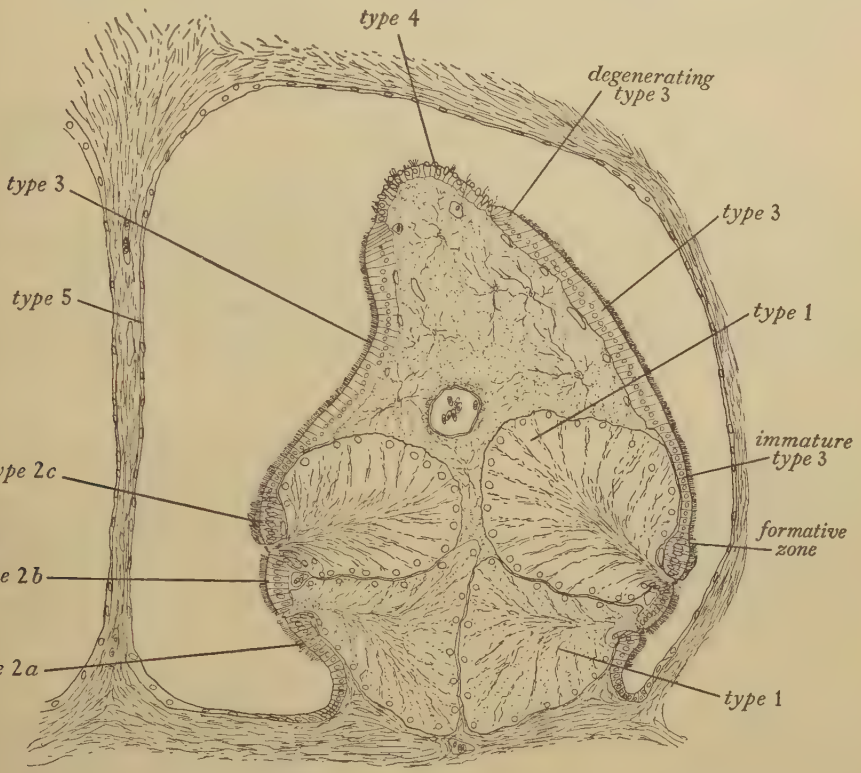

FIG. 1. Transverse section of the right anterior chamber of the endostyle.

Towards the ventral border of the type 3 zone (fig. 1), overlying the dorsal tract, the cells become gradually less tall, the nuclei lose their vesicular form and become granular and strongly basiphil, while the RNA becomes less conspicuous and loses its basal polarization (fig. 3, A). We consider that the explanation of this is to be found in the presence of a very narrow zone, constant in our material, although not shown in Sterba's figures, in which the ciliation is interrupted (figs. 1; 3, C). At this point, which sharply separates the ventral edge of the type 3 zone from type 2*c*, there is a crowding of nuclei, and we have observed a mitotic division occurring there (a very rare event in the endostylar epithelium, in our experience). We are therefore inclined to interpret this as a formative zone, from which new cells are added to the type 3 epithelium, the characteristics of the smaller cells being a consequence of

their immaturity. There is reason for thinking that a corresponding degeneration occurs at the upper border of the type 3 zone, where it adjoins type 4 (fig. 1), for cells at that point are commonly found to be losing their nuclei (fig. 2, E), while the ciliation becomes very irregular.

Bound iodine is found both in the endostylar cells and also in some of the secretion which lies in the lumen of the organ. It is conspicuous in the type 3 epithelium (fig. 3, D), being most abundant over the region of the mature cells; there is some reduction in its concentration in the degenerating cells, and a regular and marked reduction in the immature ones (fig. 3, D), amounting sometimes to virtual disappearance in the immediate neighbourhood of the formative zone. The limits of its distribution within the cells themselves are doubtless obscured by random scatter, but, making allowance for this, there is evidence that the main centre of its concentration is towards the cell apex, and that it extends at least part of the way towards the nucleus and also outwards over the cilia.

In seeking a cytological basis for thyroidal biosynthesis one might expect, by analogy with higher forms (Roche and Michel, 1955), to find a PAS-positive and colloid-like secretion associated with this bound iodine, and it is thus significant that the whole of the type 3 epithelium is, in fact, characterized by a rich production of such material. The distribution of it shows some variation in detail, even between opposite (and hence morphologically similar) sides of the same section, but we believe that it is possible to discern a relatively simple pattern.

In the mature type 3 cells this material, which we propose to call the thyroidal prosecretion, arises basally, in a region adjacent to the RNA concentration, where there is often an appearance of vacuolation (fig. 4, B). It forms as small granules or threads which give an intense PAS-positive reaction (fig. 4, A, E); these may run together into strands, or may give rise to

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FIG. 2 (plate). A, the plug of the ventral tract is central; 2*a* cells to the left, 2*b* to the right. A gradient of thyroidal granules extends from left to right in the upper part of the 2*b* cells. Note the presence of PAS-positive material in the ventral tract and its plug, and its absence from the edge of the dorsal tract, seen on the right. (PAS.)

B, the plug of the dorsal tract is to the left, type 2*c* and immature type 3 cells extend to the right. Thyroidal granules are abundant in type 2*c* (compare fig. 5, B), and the initial phase of the prosecretion is visible as darker inclusions in type 3 (compare fig. 4, E). (PAS.)

C, the plug of the ventral tract is to the right, and type 2*a* cells to the left; columnar type 5 cells extend upwards from the latter. (Azan.)

D, similar to C, but showing the accumulation of prosecretion at the bases of the type 5 cells. (PAS.)

E, degenerating type 3 cells, showing the loss of nuclei. (Azan.)

F, type 3 cells; compare with figure 4, B. (Azan.)

G, type 3 epithelium; immature cells lie to the left, and, in comparison with the mature cells to the right, show the abrupt transition to the accumulation of large amounts of prosecretory material. (PAS.)

H, mature type 3 cells; compare with fig. 4, A. (PAS.)

I, type 4 epithelium. Note the small prosecretory inclusions, one of which is being extruded in some cytoplasm (compare fig. 6, A); prosecretion is also accumulating basally. (PAS.)

J, same as I. Note the extrusion of nuclei and cytoplasm from the surface. (Azan.)

K, type 5 epithelium. Thyroidal granules are visible; compare with fig. 6, B. (PAS.)

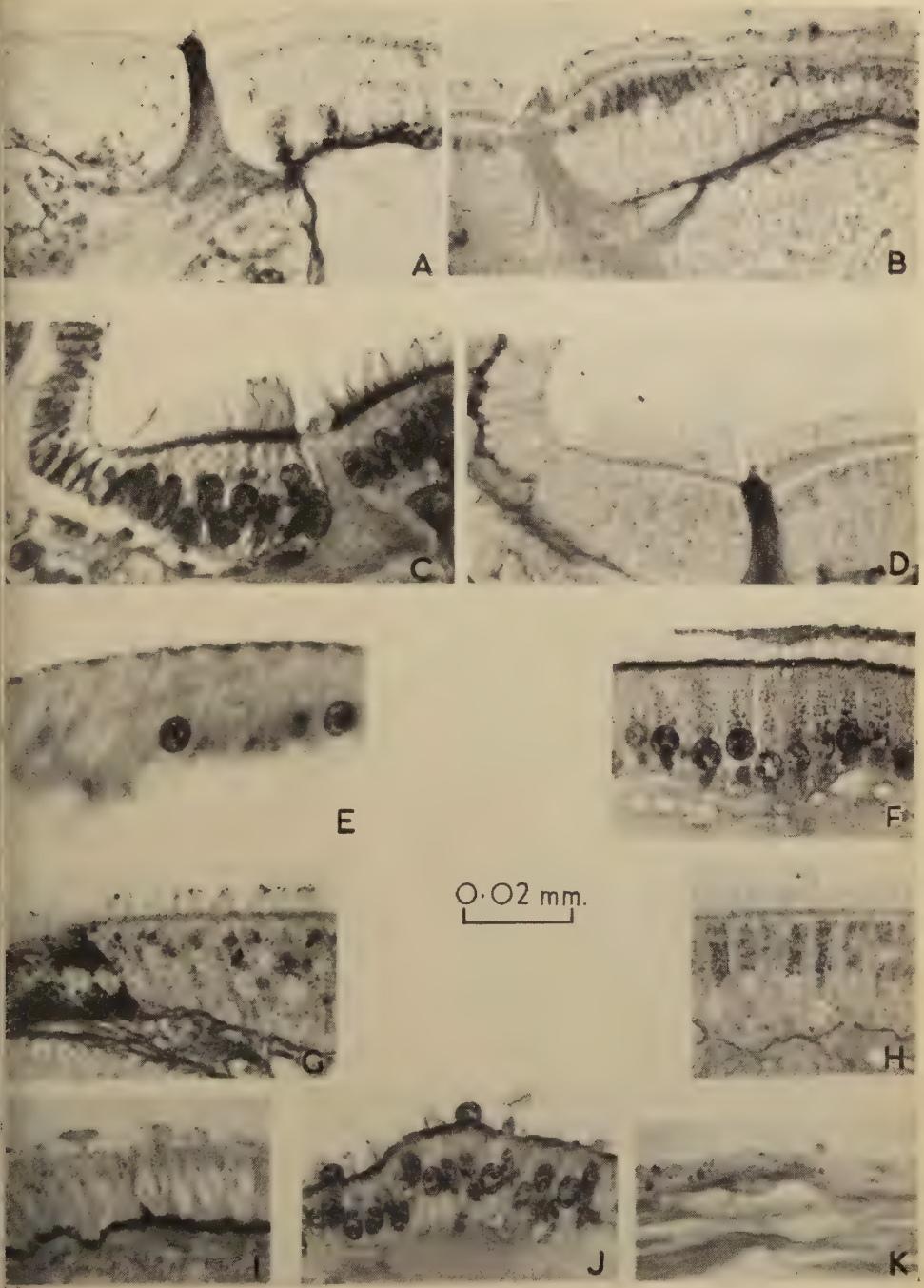

FIG. 2

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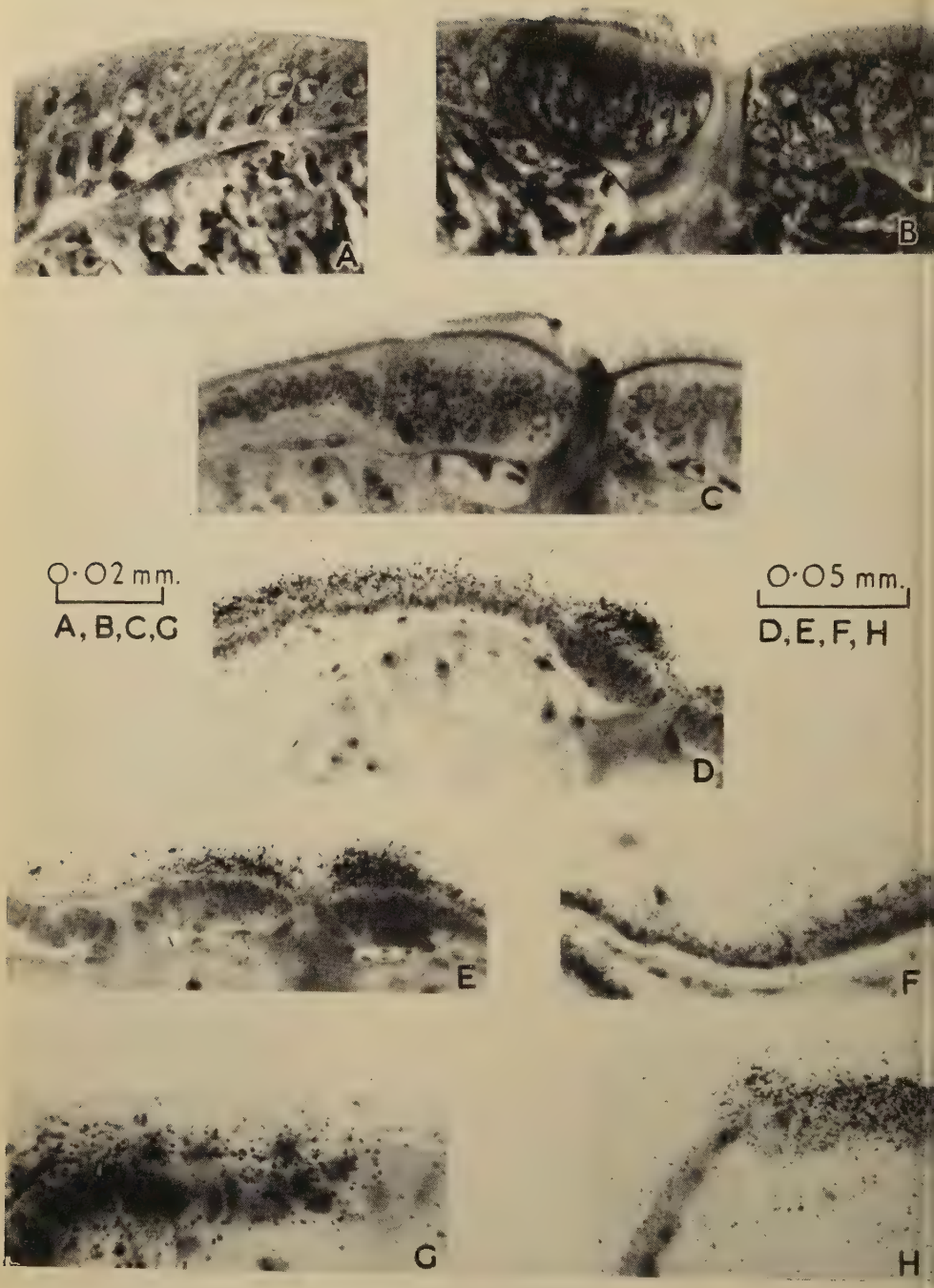

FIG. 3

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ring-like figures in which a PAS-positive surface layer seems to enclose a non-staining core. They pass up alongside the nucleus towards the apex of the cell, the cytoplasm of which may come to contain large numbers of them distributed throughout its extent; their distribution, however, does not agree with that of the bound iodine, for they are often especially conspicuous below the nucleus, where there is little or no trace of it.

The explanation of this lies in the fact that the initial phase of the prosecretion is transformed in the upper part of the cell into a second phase which it will be convenient to distinguish as thyroidal granules (figs. 2, H; 4, A, E), and which differ in being homogeneous instead of ring-like, in having a more regular form, and in often (but not always) giving a somewhat paler PAS response. These granules vary considerably in size, evidently as a result of growth, the smaller ones being more apical, while the larger ones lie immediately above the nucleus or, occasionally, immediately below it. These two phases of the prosecretion intermingle, and individual elements clearly grade into each other in shape and size; ring-like figures, for example, may develop some staining reaction in their cores so that they approximate in appearance to the granules (fig. 4, A, E).

The localization of the thyroidal granules in the upper part of the cells clearly agrees closely with that of the bound iodine, and their direct relationship with the latter is convincingly shown by a similar agreement in other parts of the epithelium, to be demonstrated below, and by a consideration of the special conditions which obtain at the upper and lower ends of the type 3 zone. At the latter (fig. 4, E), which we have interpreted above as an area of immature cells (p. 395), there is a progressive diminution in the number and size of the thyroidal granules which significantly parallels the reduction in the amount of bound iodine (see p. 396). There are also large accumulations of

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FIG. 3 (plate). A, type 3 epithelium. The mature cells to the left show RNA concentrated at their base (compare fig. 4, B); the immature cells to the right show a reduction in the amount of RNA and a tendency for it to be distributed above as well as below the nucleus. The cells of the dorsal tract, below, show abundant and diffuse RNA. (Pyronin methyl green.)

B, type 2*b* to the right type; 2*c* to the left, both groups show a diffuse RNA distribution, this being much more prominent in 2*c*. (Pyronin methyl green.)

C, type 2*b* to the right, 2*c* to the left. The 2*c* cells have matted cilia; they are succeeded to the left by the narrow formative zone, where the cilia are interrupted, and then by the ciliated immature type 3 cells. (Azan.)

D, autoradiograph. The plug of the dorsal tract lies to the right. To the right of it lie a few type 2*b* cells. To the left extend type 2*c*, immature type 3, and mature type 3. The outline of the dorsal tract is seen below the iodinated epithelium, and is free of iodine.

E, autoradiograph. The plugs of the dorsal and ventral tracts lie to the right and left respectively. Iodine is conspicuous in the type 2*c* cells to the right, is diminished in type 2*b* in the centre, and is virtually absent from type 2*a* to the left.

F, autoradiograph, showing iodine in the type 5 epithelium at the base of the median septum. Compare fig. 6, B.

G, autoradiograph. A few type 3 cells are on the extreme right, and are free of iodine; the remainder of the cells are type 4, and show signs of the presence of iodine.

H, autoradiograph. Type 3 cells above showing an abundance of iodine, which diminishes centrally over the degenerating cells; type 4 cells below and to the left appear to show no iodine.

the initial phase of the prosecretion, which often forms colloid-like masses at the bases of the cells, while in extreme cases the cell bodies may appear to be almost fully charged with it (fig. 2, G). The boundary between this condition and that of the normal type 3 cells may be a sharp one, and probably coincides with the equally abrupt loss of polarization of the RNA (fig. 3, A); in the smaller cells this tends to be diffused close around the nucleus, and it is likely that the prosecretion, which we believe to arise in close relation to this, has

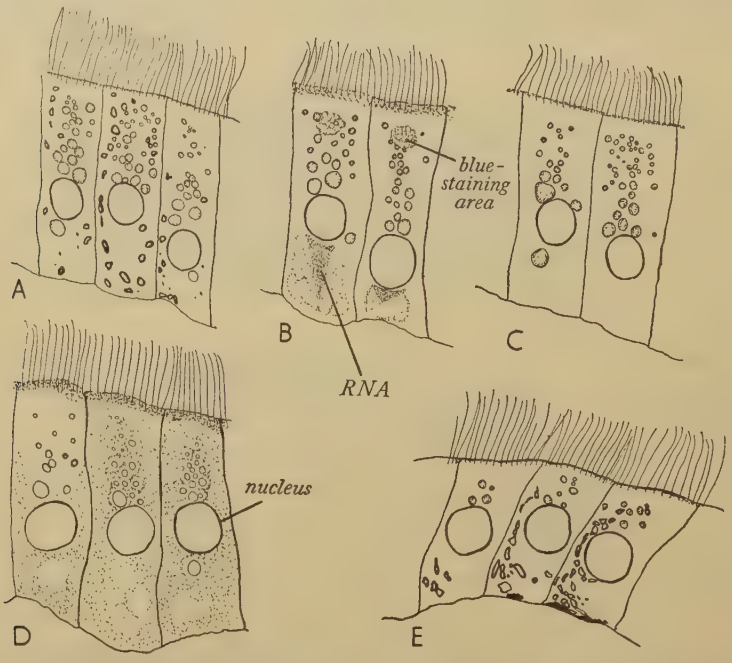

FIG. 4, A, type 3 cell, PAS. B, ditto, Azan. C, ditto, ferric-ferricyanide. D, ditto, Fontana. E, immature type 3 cells, PAS. The initial phase of the prosecretion is shown unstippled and with a heavy outline; the thyroidal granule phase is shown stippled and with a lighter outline (except in D, where it is unstippled).

a less localized origin in such cells and is therefore more widely diffused through the cytoplasm. Some reduction of thyroidal granules occurs also in the degenerating cells at the upper end of the epithelium, and here, although less frequently, colloid-like masses of prosecretion may be found at the cell bases. Such masses, both in the immature and degenerating zones, are not associated with iodine images, and, as we have seen, the apices of these cells actually show a reduction of iodine as compared with the mature type 3 cells. We shall suggest below (p. 400) a possible explanation of these conditions.

The close coincidence between the distribution of the bound iodine and of the thyroidal granules at once suggests that the latter may be related to thyroid colloid, and we have examined this possibility by applying to them some appropriate histochemical tests, as follows.

Coloration. It is well known that a brown pigment develops in the endo-

stylar epithelium, appearing first in larvae of about 25 mm (Sterba, 1953). We find this to be located within the thyroidal granules (but not in the initial phase of the prosecretion), and to be especially characteristic of the larger (juxtanuclear) ones; it is less apparent in the more apical ones, and its development appears to be progressive, for it is very restricted in amount in the immature cells, in which only one or at most a few granules commonly show it. The coloured granules are bleached within 24 hours by 10% hydrogen peroxide, are colourless after treatment with Nile blue sulphate by the method of Hueck, and give a dark yellow response to the Fontana ammoniacal-silver test (fig. 4, D). We therefore consider it probable, although not certain, that the pigment is a melanin, for the Fontana reaction is suggestive of a weakly-positive response associated with a very low concentration of the reacting substance. The progressive development of the brown colour of the granules, together with their growth, implies that they have a considerable permanence, and that the pigment itself may be a by-product indicative of maturation or of ageing; from the point of view of thyroidal function, the presence of a melanin could perhaps be interpreted as a consequence of specialized tyrosine metabolism.

Ferric-ferricyanide reaction. The granules, but not the initial phase, are coloured blue by this test (fig. 4, C). The reaction certainly extends from the larger granules to the more apical ones, although it is difficult to say whether quite all of those revealed by the PAS procedure are coloured. A wide range of substances may be expected to respond positively to this test, but thyroid colloid is included amongst these, and it has, in fact, been recommended for the identification of the latter (Lille and Burtner, 1953; Fisher, 1953). In the present instance the interpretation is admittedly complicated by the fact that melanin is also known to give a positive response, but the granules do, in fact, respond even in specimens in which they lack the brown coloration.

Fluorescence. The thyroidal granules, but not the initial phase of the prosecretion, show a yellow fluorescence in material which has been sectioned after mercuric-formaldehyde fixation and paraffin embedding, and this property does not depend upon the presence of the brown pigment, although the latter may influence the actual colour. Thyroid colloid shows blue fluorescence, and thyroid cells yellow or white, depending on the fixative used, and these properties have been attributed to the presence of proteins (Dempsey, 1944). We cannot press the comparison very far, but, as will be shown below, full development of thyroidal function is strikingly correlated throughout the endostylar epithelium with the presence of fluorescent granules.

Azan staining. The initial phase of the prosecretion is not detectable, except perhaps occasionally as faintly blue areas which are not really distinguishable from the cell background. The thyroidal granules tend to stain predominantly with orange G (fig. 2, F), the more apical ones, however, being rather indistinct, with a tendency to a bluish reaction. A constant feature is the presence towards the apex of the cell of a distinctive blue-stained area (fig. 4, B) which may appear homogeneous or may give a faint impression of granulation;

with other techniques this area (fig. 4, A, C) can be seen to be associated with separate granules which sometimes, especially in ferric-ferricyanide preparations, colour more weakly than do the more basal ones, and which are often particularly small. The condition could be interpreted as indicative of activity and transformation, and it certainly contrasts with the more static appearance of the large juxtanuclear granules; it is thus suggestive that thyroid colloid has been said to colour with orange G or azocarmine in resting glands, and with aniline blue in activated glands (Pickford, 1953; Dempsey, 1954).

The above responses are not specific; granules which accumulate at the base of the gill lamellae, for example, react similarly, although they arise differently and have no association with iodine. In the light of all the facts, however, we feel justified in drawing the following conclusions:

1. Full thyroidal activity in the mature type 3 cells includes storage of the hormone, as well as iodination, both of these processes taking place in the upper (distal) part of the cell body. This activity is correlated with the production, in close relation to the basal concentration of RNA, of a PAS-positive prosecretion which thus seems likely to be the homologue of the colloid secretion of thyroid follicle cells; some distinctive properties of its granular phase lend support to this view, but are not sufficiently specific to be decisive.

2. The distribution of the granules corresponds spatially and quantitatively with that of the bound iodine, and they thus provide a cytological basis for hormonal storage. The stability, growth and development of pigmentation which they show may be a result of the accumulation either of a reserve of material for thyroidal biosynthesis, or of by-products of thyroidal metabolism, or of both. There is as yet no evidence to determine the degree of their permanence, nor the rate at which they are depleted during the activity of the cells. It is of interest, however, that Sterba (1953), from a somewhat different viewpoint, has attributed a storage function to them.

3. The reduction of bound iodine in the immature (and, to a less extent, in the degenerating) cells indicates a corresponding reduction in hormonal storage but not necessarily in iodination, since it is theoretically possible for iodination to take place and to be followed by the immediate discharge of the product, in which case little or no image would be found in autoradiographs. It would seem more likely, however, for the thyroidal function of these cells to be reduced as a whole, and for both iodination and storage to be simultaneously diminished; the tendency for the initial phase of the prosecretion to accumulate in such cells could then be explained as a result of the overproduction of material which they are unable to utilize owing to their reduced biosynthetic activity.

These conclusions are not exhaustive, and it is clear that extended study of the type 3 cells under different conditions of activity will be needed before the secretory cycle can be fully elucidated. One matter, for example, which is at present obscure is the question of the form in which the hormone

is finally discharged. Here we can only say that while PAS-positive material can be traced up to the cell border as though it might be expelled, it does not seem to occur there with sufficient regularity to account for the very constant distribution of bound iodine over the cilia, while visible secretion associated with the latter is irregular and sometimes very scarce. Some investigators have commented on the difficulty of finding clear evidence for the discharge of secretion from the endostyle cells, and have suggested that it may pass into a non-staining condition at this stage. We are at present inclined to favour this explanation and to suppose that the iodinated product leaves the cell in a fluid and non-staining form which becomes adsorbed to the cilia. Our assumption is that the more apical of the thyroidal granules are closely involved in this final stage, either by the release of material for iodination or by the release of the stored hormone itself, and that the blue-staining area (p. 399; fig. 4, B) is the centre of this activity. The initial phase of the pro-secretion also extends up to the cell apex, however, and we have no evidence which would exclude the possibility that this might also give rise to some definitive secretion without passing through the granule stage. Further information on this whole matter is clearly desirable, particularly in view of the recent demonstration of the presence in the endostyle of a catheptic protease which could be regarded, by analogy with higher vertebrates, as available for hydrolysis of thyroglobulin (Clements and Gorbman, 1955).

Type 1 cells. These are the cells which constitute the dorsal and ventral glandular tracts (fig. 1), their distal ends being fused to form a plug-like structure through which their secretion is discharged into the endostylar lumen. We agree with Gorbman and Creaser (1942) in finding no bound iodine in them, and because of this, and because of the well-established fact that the complete disappearance of the tracts is one of the earliest signs of metamorphosis, we conclude that they are not concerned with thyroidal function, but that they produce the mucus-like secretion which has been considered as an important part of the feeding mechanism (Dohrn, 1886; Kieckebusch, 1928; Newth, 1930; Young, 1950).

In agreement with this view, the cytological characteristics of the type 1 cells differ considerably from those of type 3. In our mercuric-formaldehyde preparations their secretory product appears as masses, negative to muci-carmine, lying in irregular spaces which, according to Sterba (1953), are intracellular. This secretion stains weakly with aniline blue, is negative to the ferric-ferricyanide test, gives only a very pale PAS-positive response, and shows no brown colour or yellow fluorescence. The cells themselves differ from type 3 in the diffusion of a strong RNA reaction throughout the cytoplasm (fig. 3, A, B), in the presence (also throughout the cell body) of well-defined and filamentous mitochondria (see also Hensel, 1932, and Sterba, 1953), and in the fact that the vesicular nuclei are uniform in staining reaction, and possess a larger nucleolus.

The precise nature of the secretory product of these cells is obscure, and we have nothing to add at this stage beyond drawing attention to some

differentiation between the dorsal and ventral tracts, already inferred by Hensel (1932) on other grounds. The cytoplasm of the ventral tracts differs from that of the dorsal ones in showing a greater affinity for aniline blue, while their secretion contains delicate and irregular masses of a substance which gives an intense PAS-positive reaction (fig. 2, A). The nature of this material is not clear, for it does not exactly correspond in form with the secretory product that is stained by aniline-blue, but it is presumably a component of it.

Type 2 cells. These are tall columnar cells, strongly ciliated, which lie immediately adjacent to the openings of the tracts. Gorbman and Creaser (1942) refer to occasional faint records of bound iodine in them, but we find the association of iodine with certain of the cells to be clear-cut and very constant, and attach great importance to it in our analysis. For this reason we find it essential to subdivide the cells into the three categories, 2*a*, 2*b*, and 2*c* (fig. 1).

The cells of type 2*a* lie ventrally to the ventral tracts, with which they have a special relationship. In transverse section each tract is horseshoe-shaped (fig. 1), and new cells are added to it at the ventral tip from the type 2*a* epithelium with which it is continuous; thus the more ventral cells of each tract are the younger ones, while the more dorsal are older, and a tendency for nuclei to disappear in the latter region suggests that the cells ultimately degenerate towards the tip of the dorsal arm of the horseshoe. This relationship emphasizes the underlying unity of pattern in this complex epithelium, for a similar situation has been seen to exist in the type 3 epithelium, with the new cells being added ventrally and degeneration occurring dorsally (see p. 395).

No alimentary secretion appears to be produced within the 2*a* cells (Sterba, 1953), and in correlation with this an RNA reaction is negligible or absent, although filamentous mitochondria, similar to those of type 1, are plentiful and well defined. The nuclei are granular (fig. 2, C), staining deeply with toluidine blue, and have only a small nucleolus; a transformation into the vesicular type with large nucleolus is readily observable in those cells which are passing into the ventral tract. No significant amount of bound iodine is associated with the 2*a* cells, and their characteristics can be regarded as in keeping with the status of immature alimentary cells. Nevertheless, we consider them also to be very slightly influenced by thyroidal differentiation, this influence being indicated by the presence of variable amounts of material resembling prosecretion, and by the occasional formation of a few PAS-positive granules above the nucleus (figs. 2, A; 5, D). The latter we interpret as rudimentary thyroidal granules, for, although they lack the special properties of the fully-developed granules (they do not fluoresce, for example, and are negative to the ferric-ferricyanide test), they can be traced as a continuous series into the type 2*c* cells (see below).

The cells of type 2*b* extend between the openings of the ventral and dorsal tracts (fig. 1) and have exactly the same relationship to the ventral edge of the latter as have the 2*a* cells to the former; in other words, they are immature alimentary cells from which cells are recruited to the dorsal tracts, and their

form, organization, and ciliation is largely similar to that of the type 2*a* cells. Mitochondria are conspicuous, as in the latter, and RNA is negligible or absent, except in the more dorsal cells (i.e. those adjacent to the opening of the dorsal tract), where a definite positive reaction may be visible in the neighbourhood of the nucleus (fig. 3, B). This latter detail is significant in

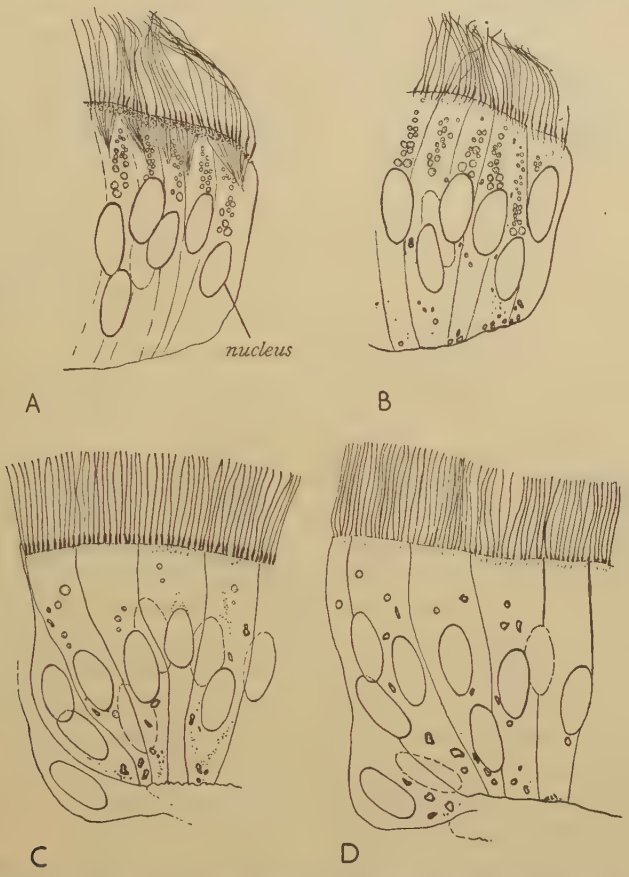

FIG. 5, A, type 2*c* cells, Azan. B, ditto, PAS. C, type 2*b* cells, PAS. D, type 2*a* cells, PAS. The figures may be compared with the right side of fig. 1, the more ventral cells of each group being to the right. Conventions as in fig. 2.

view of the fact that bound iodine is also regularly associated with these particular cells, partly in the apical cytoplasm and even more over the cilia (fig. 3, E). Thyroidal influence thus becomes more pronounced in this part of the epithelium, and this is reflected in the thyroidal granules, which increase in number from the more ventral to the more dorsal cells (figs. 2, A; 5, C). Particularly significant is the fact that a few of these in the most dorsal cells are fluorescent and give a positive ferric-ferricyanide response; slight indications of brown coloration are also sometimes visible. Such details are most important for our analysis, for they clearly justify the thyroidal significance which we

have attached to these characteristics in our account of the type 3 epithelium. We conclude, then, that a gradient of increasing thyroidal differentiation can be detected in the 2*a* and 2*b* cells, with thyroidal function becoming clearly defined in the more dorsal cells.

The cells of type 2*c* lie dorsally to the opening of the dorsal tract, and differ from 2*a* and 2*b* in showing consistent and much more pronounced thyroidal activity. This is doubtless correlated with their reduced alimentary significance, for as a result of their position they have no direct continuity with the type 1 cells (fig. 1), while in development their relationship is primarily with type 3 (Hensel, 1932). Long cilia are present, as in types 2*a* and 2*b*, but they are always matted together at their tips to form a conspicuously stained mass (figs. 3, C; 5, A, B). Kieckebusch (1928), while not mentioning this particular feature, formed the opinion that these cilia were possibly different from those of the remaining type 2 cells (i.e. our types 2*a* and 2*b*) in being non-motile, like those of type 3. The nuclei of the 2*c* cells resemble those of 2*a* and 2*b*, but in other respects there are some significant differences. The mitochondria, although clearly defined, are much fewer in number, while RNA, diffused around the nucleus, is much more conspicuous (fig. 3, B). In such features the cells show a transition to the organization of the type 3 cells, and this is clearly demonstrated by the presence of abundant thyroidal granules (figs. 2, B; 5, B). These show a further development of the gradient of differentiation already noted in the other type 2 cells, for they now have all of the properties of the type 3 granules, including brown coloration, fluorescence, orange G staining, and positive ferric-ferricyanide response, and in exact correlation with this there is a regular association of bound iodine with these cells, in noticeably greater amounts than over the 2*b* cells (fig. 3, E).

One difference between the thyroidal activity of the 2*c* and 3 cells is that in the former, as also in 2*b*, there often seems to be relatively less iodine in the cytoplasm and more over the cilia (fig. 3, D, E). This suggests that in the type 2 cells there may be a more rapid turnover of iodine and less storage of the bound product, and we are inclined to associate this with the absence from them, in Azan preparations, of the apical blue-stained area which is a regular feature of the type 3 cells, its place being taken by more separate granules (fig. 5, A). This seems to imply some difference in the course of events at the cell apex, but we are unable to comment on this until there has been some clarification of the mechanism of discharge of the iodinated secretion. It would seem likely, however, that the high concentration of iodine over the type 2*c* cilia may be connected with their fusion and their alleged non-motility, and that considerable adsorption must take place there.

Type 4 cells. This epithelium (fig. 1) forms the apex of the triangle of which the type 3 epithelium forms most of the two sides, but towards the extreme anterior end of the endostyle it disappears completely. Although it thus constitutes one of the less conspicuous features of the endostylar epithelium, its cells have presented somewhat of a problem in the literature because, while they appear to make a major contribution to the thyroid follicles of the adult

at metamorphosis (Marine, 1913; Leach, 1939; Sterba, 1953), they have been said not to bind iodine and to lack, therefore, any thyroidal properties in the larva (Gorbman and Creaser, 1942). They are lower and often more slender than the type 3 cells (fig. 6, A), and Leach (1939) found them to be unciliated in older specimens of *Ichthyomyzon fossor*; in our material, however, certain of the cells, even in older larvae, are clearly ciliated (fig. 2, J). At the endostylar duct the type 4 epithelium becomes continuous with the pharyngeal lining, and the two epithelia resemble each other in possessing granular and basiphil nuclei. Diffuse RNA and well-defined filamentous mitochondria are present in both; these, it will be noted, are characteristics which we have already associated with the alimentary cells of the glandular tracts, and their

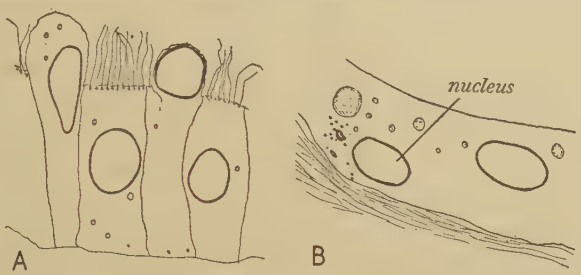

FIG. 6, A, type 4 cells, PAS. B, type 5 cells from the ventral edge of the median septum, PAS. Conventions as in fig. 2.

presence in the pharyngeal epithelium is in agreement with this interpretation. The pharyngeal cells produce a mucous secretion which gives a positive response to mucicarmine. The type 4 cells differ in lacking this secretion and in possessing a few very small PAS-positive granules (figs. 2, I; 6, A), some of which resemble the thyroidal granules of the type 3 cells in being positive to the PAS and ferric-ferricyanide tests, in showing a yellow fluorescence, in being brown, and in staining with orange G. It is significant that these characteristics are particularly conspicuous in the immediate neighbourhood of the type 3 cells, as though, like type 2, they are influenced by the extension of thyroidal differentiation from that zone. Autoradiographs commonly show no sign of bound iodine in the type 4 region, and in this respect, as Gorbman and Creaser (1942) found, there is often a sharp contrast between the type 4 cells and the adjacent type 3 (fig. 3, H). However, the undoubted presence in this epithelium of a few inclusions of thyroidal type has led us to scrutinize it very carefully, and in certain specimens which had been removed directly from mud to radioiodine solution we have found slight but probably significant indications of bound iodine in some of the cells (fig. 3, G). We are therefore inclined to interpret the type 4 epithelium as being closely related to the pharyngeal epithelium, but as differing from the latter in not producing any mucus, and in possessing a rudimentary capacity for thyroidal function which may, perhaps, be more apparent under natural conditions than in the laboratory. The initial phase of the prosecretion is sometimes very well developed in this epithelium, in which it may accumulate as colloid-like masses at the

bases of the cells (fig. 2, I). Its presence is in agreement with our general interpretation in illustrating the tendency for such material to be prominent in areas where thyroidal function, although present, is reduced in amount (p. 400).

Another feature of the type 4 cells which has aroused discussion is the peculiar way in which nuclei and cell bodies are extruded from it in large numbers (figs. 2, I, J; 6, A), replacement taking place by amitotic division (Sterba, 1953). We find that the thyroidal granules may be extruded with the cytoplasm (fig. 2, I), so that the process is essentially one of holocrine discharge, although we have never observed this taking place in any other part of the epithelium. It is perhaps significant that the discharge of cells or nuclei from the thyroidal epithelium into the follicular lumen has been reported both in adult lampreys and in fish (Olivereau, 1952; Gorbman and others, 1952), and has been regarded in them as a form of holocrine secretion, for this suggests that the behaviour in this respect of the type 4 cells, like their rudimentary thyroidal function, may be foreshadowing their future behaviour in the adult. Even if this suggestion is correct, however, the significance of the holocrine discharge remains obscure, although it might, perhaps, have been part of the mechanism by which in the early vertebrates a gland initially adapted for the intracellular storage of its product was transformed into one adapted for release of the secretion into enclosed follicles.

Type 5 cells. These are the non-ciliated cells which line the outer wall of the endostylar chambers (fig. 1). Owing to their extreme flattening it is impossible to present a detailed analysis of the pattern of cell organization comparable with that given for the other types. It can, however, be said that Kieckebusch (1928), who, like other observers, had noted the presence of brown granules in the endostylar epithelium, found them to occur sporadically in these cells, and we have observed that parts of the type 5 epithelium contain many PAS-positive and ferric-ferricyanide positive inclusions (figs. 2, K; 6, B) which show a yellow fluorescence, sometimes stain with orange G or with aniline blue, and which are clearly to be regarded as thyroidal granules; the initial phase of the prosecretory material is also present in these regions (fig. 6, B). The presence of RNA is identifiable, notably in the dorso-lateral area of the epithelium, but mitochondria are not resolvable in our preparations. Bound iodine is often associated with the epithelium and, although it is difficult always to be sure whether it is in the endostylar lumen or in the cells themselves, we consider, in agreement with Gorbman and Creaser (1942), that it certainly is found within the cells in certain areas; it is particularly abundant dorso-laterally and in the epithelium which covers the median septum separating the two endostylar chambers (fig. 3, F), and a correlation of this with the presence of thyroidal secretion is very clear in some of our preparations at the ventral edge of the septum. At the point where the type 5 cells adjoin type 2*a* the former become columnar (fig. 2, C). Here thyroidal granules and bound iodine are absent, but there may be substantial accumulations of the initial phase of the prosecretory material (fig. 2, D),

illustrating again the correlation between the presence of conspicuous amounts of this and the reduction of full thyroidal function.

We conclude that thyroidal function is certainly present in this epithelium and that its cytological characteristics, in so far as they can be defined, are in agreement with our general interpretation.

Discussion

Doubts recently expressed (Gorbman, 1955) as to the homology of the ammocoete's endostyle with that of the protochordates, based on the lack of evidence of iodine-binding in the latter, have been largely dispelled by the discovery (Barrington and Franchi, 1956) that bound iodine does, in fact, occur in part of the endostylar epithelium of *Ciona*. The argument that the endostyle of the ammocoete can in any case be homologized with only a part of that of the protochordates, since it differs from the latter in communicating with the pharynx by a narrow opening instead of by a longitudinal groove, overlooks the fact that the organ develops in the ammocoete as a groove of the floor of the pharynx, with the reduced opening appearing as a secondary feature (Kieckebusch, 1928). The pharynx itself has, of course, profoundly changed during the transition from protochordates to vertebrates, muscular activity having become substituted for ciliary, but this cannot be held to weaken the homology of the pharynx of the two groups, nor does it seem reasonable that the fundamental homology of the endostyle should be weakened by its own increase in complexity.

We ourselves have no hesitation in supporting the old-established view that the history of the thyroid 'has been one in the course of which a feeding structure has been transformed into a gland of internal secretion' (Romer, 1955), for our results are fully in accord with that interpretation. They show that part of the epithelium is organized for the production of the secretion which contributes to the feeding mechanism; this must represent the persistence into the vertebrates of the primitive 'alimentary activity' of the endostyle, although the precise significance of the secretion is less well understood in the ammocoete than it is in *Amphioxus* and the Tunicata (Orton, 1913). Other parts are organized for 'thyroidal activity', which is localized in cells of a different character, and which presumably represents a later evolutionary development, although further analysis of the situation in the Tunicata is needed in order to clarify this matter. We have shown that a fundamental feature of the thyroidally-active cells is the formation in them of thyroidal granules, which, in their relation to the bound iodine, and in their staining and histochemical properties, sufficiently resemble thyroid colloid to create a strong presumption that they are homologous with it. If this presumption is correct, it follows that the biosynthesis of thyroid hormone in the endostylar epithelium must follow a course closely similar to that found in the thyroid of higher forms, but with the thyroidal granules providing a basis not only for the iodination of tyrosine but also for the intracellular storage of the colloid both in its uniodinated and iodinated form (p. 400).

The pattern of the distribution in the epithelium of the alimentary and thyroidal functions has proved to be rather complex; in particular, there are indications of gradations of activity, so that functional differentiation overlaps the boundaries between the morphologically-characterized cell-types. It therefore appears to us to be helpful, for descriptive purposes, to state the situation in terms of the embryological concept of gradient fields. From this point of view it may be supposed that an alimentary field embodies the type 1 cells, and extends also over type 2, influencing especially 2*a* and 2*b*, which have a direct developmental relationship respectively with the ventral and dorsal tracts, and which can thus be regarded as immature alimentary cells. This field is associated in types 2*a* and 2*b* with active cilia (Kieckebusch, 1928), in types 1, 2*a*, and 2*b* with abundant and conspicuous filamentous mitochondria, and in type 1 with abundant and diffuse RNA, with vesicular nuclei exhibiting a constant staining reaction, and with a somewhat ill-defined secretory product which stains with aniline blue.

Standing in contrast to this field there is a thyroidal field which embodies the type 3 cells, and extends also over type 2, so that these, from 2*c* to 2*a*, show a steadily diminishing gradient of thyroidal influence. This field is associated in type 3, and perhaps also in type 2*c*, with cilia which may be inactive (Kieckebusch, 1928), and in type 3 with very slender mitochondria, basally-polarized RNA and vesicular nuclei which exhibit a staining reaction varying from basiphil to acidophil. Particularly characteristic of the field, however, is the thyroidal prosecretion, which is found throughout types 2 and 3, but only develops into fully-processed thyroidal granules in types 2*c* and 3, and in those 2*b* cells which immediately adjoin 2*c*. The situation in the type 2 epithelia can thus be explained as a consequence of the increasingly imperfect and rudimentary development of thyroidal function in those cells which are more closely associated with the alimentary field, and the distribution of bound iodine has been seen to be in agreement with this interpretation. Thyroidal activity, associated with thyroidal granules, is also distributed over the unciliated type 5 epithelium, but is clearly reduced where this adjoins the type 2*a* cells and where it may be supposed to be influenced by the alimentary field. It can also be detected to a very limited extent in type 4, particularly in the immediate vicinity of type 3, where again the thyroidal field of the latter might be expected to exert some effect; the considerable suppression of this field in the type 4 zone is perhaps a result of the close association of the latter with the pharyngeal epithelium.

Some authors have found it puzzling that, as originally shown by Marine (1913) and Leach (1939), and recently confirmed by Sterba (1953), the type 3 cells, predominantly concerned with iodination in the larva, seem largely to dedifferentiate at metamorphosis, while the type 4 cells make a major contribution to the adult follicles. Assuming that this is correct (and Sterba has rightly emphasized the difficulty of following particular cell-types through a protracted metamorphosis), such a course of events would seem to be in logical accord with our own observations. The extracellular storage of

iodinated thyroglobulin as thyroid colloid is morphologically impossible in the endostyle. The type 3 cells, therefore, with their intracellular storage, may be regarded as adapted for the requirements of larval life, and it would be in line with the usual course of events in metamorphic life-histories for such specialized larval features to disappear. Thyroidal function is, as we have shown, quite widely spread through the epithelium, and it would not be unexpected for new thyroidal cells to be recruited from the type 4 epithelium which, as far as we can judge, appears to be relatively unspecialized. The separation at metamorphosis of the type 4 epithelium from the pharynx, and hence from alimentary influence, may well be the factor which releases its capacity for full thyroidal differentiation.

The evidence for the intracellular binding and storage of iodine within the endostylar cells is of interest in view of current doubts as to whether iodination in the mammalian thyroid takes place in the cells or in the follicular colloid (Wollman and Wodinsky, 1955), for it is clear that iodination is intracellular at the endostylar level of evolution. At present our observations give no answer to the question of the form in which the hormone is released from the cells, for although the presence of bound iodine is clearly demonstrable in the endostylar lumen in autoradiographs, this gives no clue as to the actual mode of discharge. It is clear, therefore, that more remains to be learned of the secretory processes of the endostylar epithelium, and consideration needs to be given to the possibility (Sterba, 1953) that the secretory product may pass into intercellular spaces before entering the lumen.

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Studies of Serpulid Tube Formation

I. The Secretion of the Calcareous and Organic Components of the Tube by *Pomatoceros triqueter*

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With 2 plates (figs. 1 and 2)

Summary

Calcium carbonate, in a finely granular form, is secreted into the fold of the peristomial collar by a pair of tubulo-racemose glands in the peristomium.

Most of the organic material of the tube is secreted by the ventro-lateral epithelial cells which surround the main ducts of the calcium-secreting glands. These cells are always filled with a basiphil mucigen which contains some free *a*-glycol groups. This material retains its affinity for basic dyes and for ferric iron at a low pH. The affinity for iron is retained when the pH is low enough to exclude carboxyl and phosphoric acid groups from the reaction and it is probable that the sulphuric acid groups are responsible.

The reactions of this mucigen to histochemical tests together with the similarity in behaviour of a precipitated heparin preparation make it likely that the material is a sulphomucopolysaccharide.

Introduction

POMATOCEROS tubes are composed mainly of calcium carbonate and an organic substance. These two major components are thoroughly mixed together, for after decalcification an organic tube remains which is an exact model of the calcified structure. Descriptions of *Pomatoceros* tubes have been made by Dons (1927) and by Thomas (1940), and Segrove (1941) gave an account of the young fixed stages with a description of the initial tube formation.

Any addition to the adult tube is at the anterior end from which the worm protrudes. In a protruded state the branchial crown is expanded and the collar of the peristomium folds back over the anterior face of the tube. It is in this collar fold area, therefore, that the calcium is deposited.

The problem of tube formation may be dealt with under three headings: (*a*) the nature of the calcareous component, (*b*) the nature of the organic substance, and (*c*) the mode of deposition of the calcium and organic constituents. This paper makes a contribution to (*a*) and (*b*) and elsewhere an account of the deposition in the form of a tube will be given.

Previously it was thought that in *Pomatoceros* the calcium part of the tube comes directly from the sea water (Robertson and Pantin, 1938; Thomas, 1940). Two simple saccular glands, the major subcollar glands, have been described in the brackish water *Mercierella enigmatica* Fauvel and are responsible for the secretion of calcareous tube material (Swann, 1950).

In the following account the structures described by Thomas (1940) as

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lateral sense organs are shown to be exocrine tubulo-racemose glands responsible for the production and secretion of calcium carbonate.

Material and Methods

The worms (*Pomatoceros triqueter* L.) were collected from the inter-tidal zone at Cullercoats. A healthy laboratory supply was maintained without special feeding, with a change of water every few days.

Methods for micro-anatomy. Four hours' fixation in Bouin or Zenker solutions gives a satisfactory result for the general micro-anatomy. For the elucidation of the internal structure of the calcium-secreting glands, however, only Zenker-formol (fig 2, A,) or Lewitsky's fluid (Flemming-without-acetic) (fig. 2, C) are recommended. With these a fixing period of 4 hours is adequate. Fixation in all other solutions commonly used has a devastating effect on the glands (fig. 2, B).

Histochemical detection of calcium. A neutral fixative is essential and the best results were obtained with worms which had been fixed for 3 hours in a mixture of 9 parts of absolute alcohol and 1 part of neutral formalin. Sections 10μ thick were treated with the following reagents according to the procedure recommended by the authors: (*a*) the gallic acid reagent (Cretin, 1924); (*b*) 0·5% aqueous solution of sodium alizarin sulphonate (Lison, 1936); (*c*) saturated solution of purpurin (Lison, 1936); (*d*) a saturated aqueous solution of gallamine blue (Stock, 1949).

Micro-incineration method. The worms were fixed in the absolute alcohol / formalin mixture, dehydrated, and embedded in wax. Sections cut at 6μ were mounted on glass microscope slides, absolute alcohol being used for the floating and flattening process. The sections were incinerated in a small electric furnace. The slides were placed on a glass support and put into the middle of the oven. Scott (1937) recommended a slow increase in temperature to 200° C for the incineration of tissues to avoid shrinkage of the cells. The following times are adequate for complete incineration of *Pomatoceros* sections: 0°–75° C in 8 minutes; 75° 200° C in the next 6 minutes; 200°–600°C in the next 16 minutes. It is necessary to maintain the temperature at 600° C for 5 minutes to ensure the complete incineration of the thoracic nephridia. The remainder of the tissues are totally incinerated as soon as 600° C has been reached.

Staining methods for the detection of mucus. Worms fixed in Bouin or Zenker solutions and cut at 6μ were stained with Southgate's mucicarmine or Mayer's muchaematein (Gatenby and Beams, 1950).

Histochemical detection of mucus. Worms fixed in a mixture of equal volumes of 8% basic lead acetate and 14% formalin were stained in toluidine blue 0·1% in 30% alcohol (Sylven, 1941). Sections of worms fixed in the lead acetate / formalin solution, or in acetone, were treated with periodic acid / Schiff reagent (PAS) (McManus, 1948); the procedure used was that of Hotchkiss (1948). Control sections were treated with Schiff's solution without prior oxidation. All sections were incubated with diastase before the application of the procedure.

Ferric iron uptake by tissue sections. The methods were based on that of Hale (1946). Worms were fixed in Carnoy's fluid, or absolute alcohol, or simply dehydrated in acetone. Sections (10μ) were immersed in a 5% ferric chloride solution to which was added an equal volume of either acetic acid (recommended by Hale) or hydrochloric acid. After washing in distilled water the iron taken up was demonstrated as the Prussian blue compound after immersion of the sections in equal volumes of 1·5% potassium ferrocyanide and 0·5% HCl. The slides were then washed in distilled water, dehydrated, cleared in xylene, and mounted in neutral balsam.

The Calcium-secreting Glands

There is one in a lateral position on each side of the peristomium (fig. 1, A, and B). The main duct to the exterior passes through the lateral epithelium and the gland mass is embedded in the sub-epithelial tissue.

Glands occur which are spheroids, whilst others have a major axis much larger than the minor axis and are elongated ellipsoids. In an average worm, approximately 1·5 cm long from the tip of the branchial crown to the anus, the gland has a major axis of about 100 to 150μ and a minor axis of about 80 to 120μ. In addition to this expected variation in size in different worms there is occasionally a significant difference between the two glands in a single worm.

The diameter of the main duct to the exterior is between 10 and 15μ. The lateral epithelium has a covering cuticle layer and this continues as a layer lining the main gland duct. This cuticle is thickest near the opening of the duct, becoming gradually thinner until it disappears at the junction of the main duct and the gland mass.

The gland mass is tubulo-racemose or compound tubular (fig. 2, C). Each component tubule has a single layer of cells radially arranged round a central lumen. These tubules twist in one or two directions and their ducts are interconnected with those of other tubules. The diameter of a tubule from a gland of average size is 40μ with the lumen approximately 10μ. The secreting cells are cubical with oval nuclei at the end opposite the lumen.

The calcium-secreting glands are surrounded by connective tissue, and although there does not appear to be a gland sheath there are isolated bands of smooth muscle in the connective tissue immediately surrounding the gland. These muscle bands are only found near the glands, and not elsewhere in the connective tissue.

The detection of calcium in the peristomium. Initial attempts to locate calcium in the peristomium by histochemical methods were unsuccessful. After the calcium content of the calcium-secreting glands had been demonstrated by the incineration process, the histochemical methods were tried again. After purpurin the calcium in the ducts of the gland gave a very faint pink lake, whilst the calcium content of the cells did not react. Compared with the red lake obtained after staining bone sections and calcium carbonate smears, the positive result with the duct contents was unconvincing. After staining with gallamine blue a light blue lake was produced with the calcium in the ducts.

In sections stained with toluidine blue the calcium content of the ducts and cells was seen to be in the form of very small granules, which were not birefringent.

A number of complete series of incinerated sections of the peristomium were examined. The results in each were essentially the same with the exception of the cells of the thoracic nephridia. A heavy residue of white calcium ash was found in the ducts and cells of the calcium-secreting glands (fig. 2, D). It is concluded from a study of incinerated sections that the calcium concentration in the ducts is essentially the same as that in the gland-cells. The mucus-secreting cells of the ventral and lateral epithelium, surrounding the opening of the gland, contained more calcium ash than any other tissue with the exception of the glands. In some worms the cells of the thoracic nephridia had a high concentration of calcium ash, whilst in other specimens the amount was very small. It is appreciated that whatever discipline is enforced by the observer such visual comparisons are only crude estimates. For this reason only substantial and obvious differences in ash concentrations have been noted.

After incineration a section of the gland was protected with a film of celloidin and subjected to the purpurin method. The lake formed with the calcium of the ducts and cells was easily recognizable and characteristic. This excludes the possibility of the concentration of the calcium in the gland being too small to produce a visible lake in the unburnt sections. The difference in colour intensity of calcium in the duct after staining normal and incinerated sections is appreciable. From this it appears that some organic substance is inhibiting the reaction, because the colour is not only less intense than in incinerated material but also when comparison is made with comparable amounts of pure calcium carbonate or bone. The matrix in the ducts of the gland inhibits the lake-forming reaction to a certain extent, but the reaction with the calcium of the gland-cells is inhibited completely. The sensitivity of a reaction of this type is difficult to assess, dependent as it is on the state of the calcium and nature of the matrix, and probably on the precise nature of the relationship between the calcium and the matrix.

For *Pomatoceros* calcium-gland tissue the lake methods give poor results and it appears that there is some inhibiting action by the matrix on the duct content, and an additional or entirely new inhibitor on the calcium of the gland-cells.

The Organic Component of the Tube

The calcium-secreting glands open to the exterior in the middle of a large mucus-secreting region. This is seen on the ventro-lateral surface on each side of the peristomium, and in serpulids other than *Pomatoceros* it has been called the ventral shield. The mucus-cells in the ventro-lateral epithelium are

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Fig. 1 (plate). A, a horizontal section through the branchial crown, thorax, and anterior region of the abdomen. 8 μ. Heidenhain's iron haematoxylin and orange G.

B, an oblique section through the peristomium, showing the calcium-secreting gland of one side. 6 μ. Hansen's trioxyhaematein and orange G.

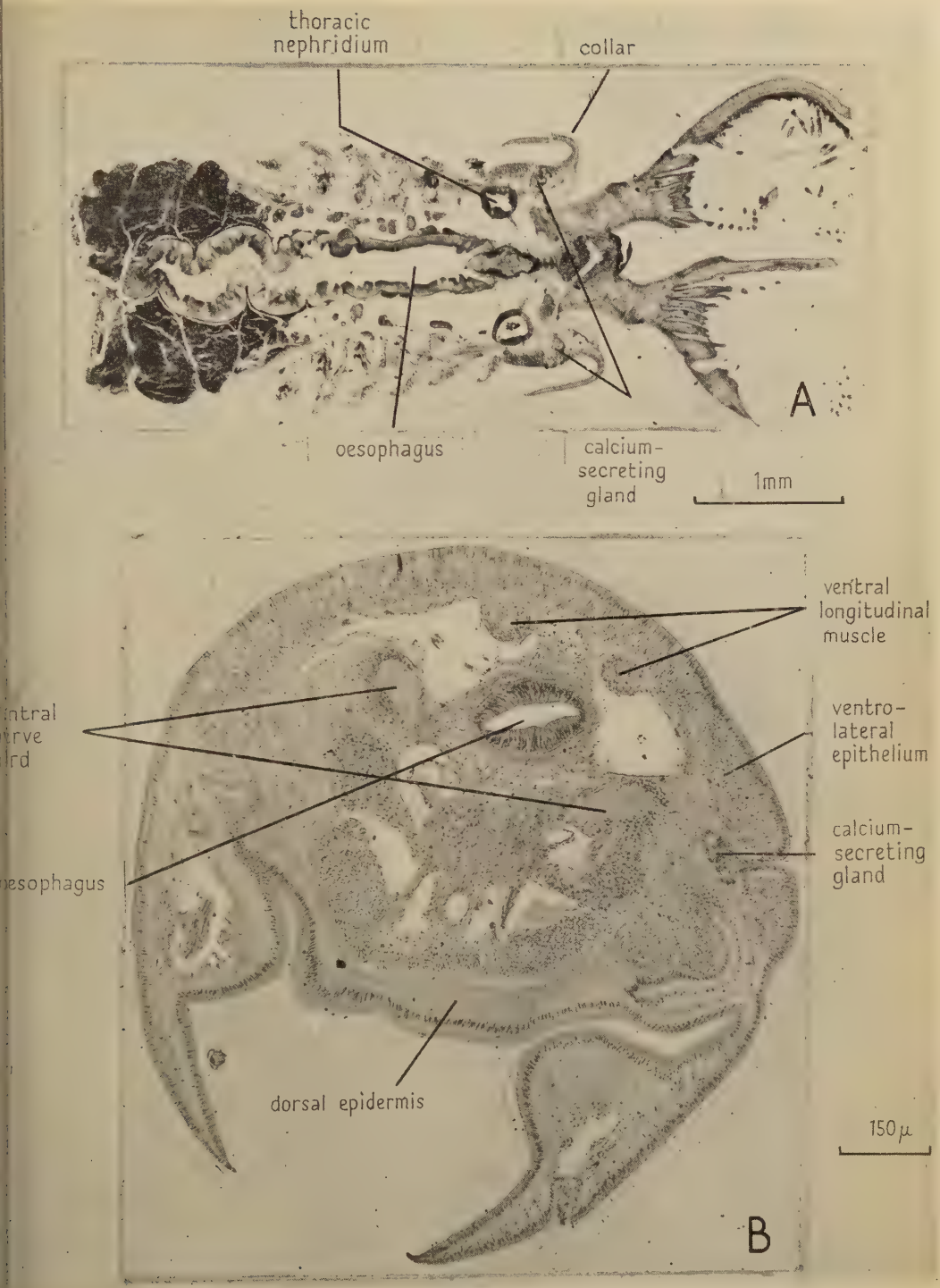

FIG. 1

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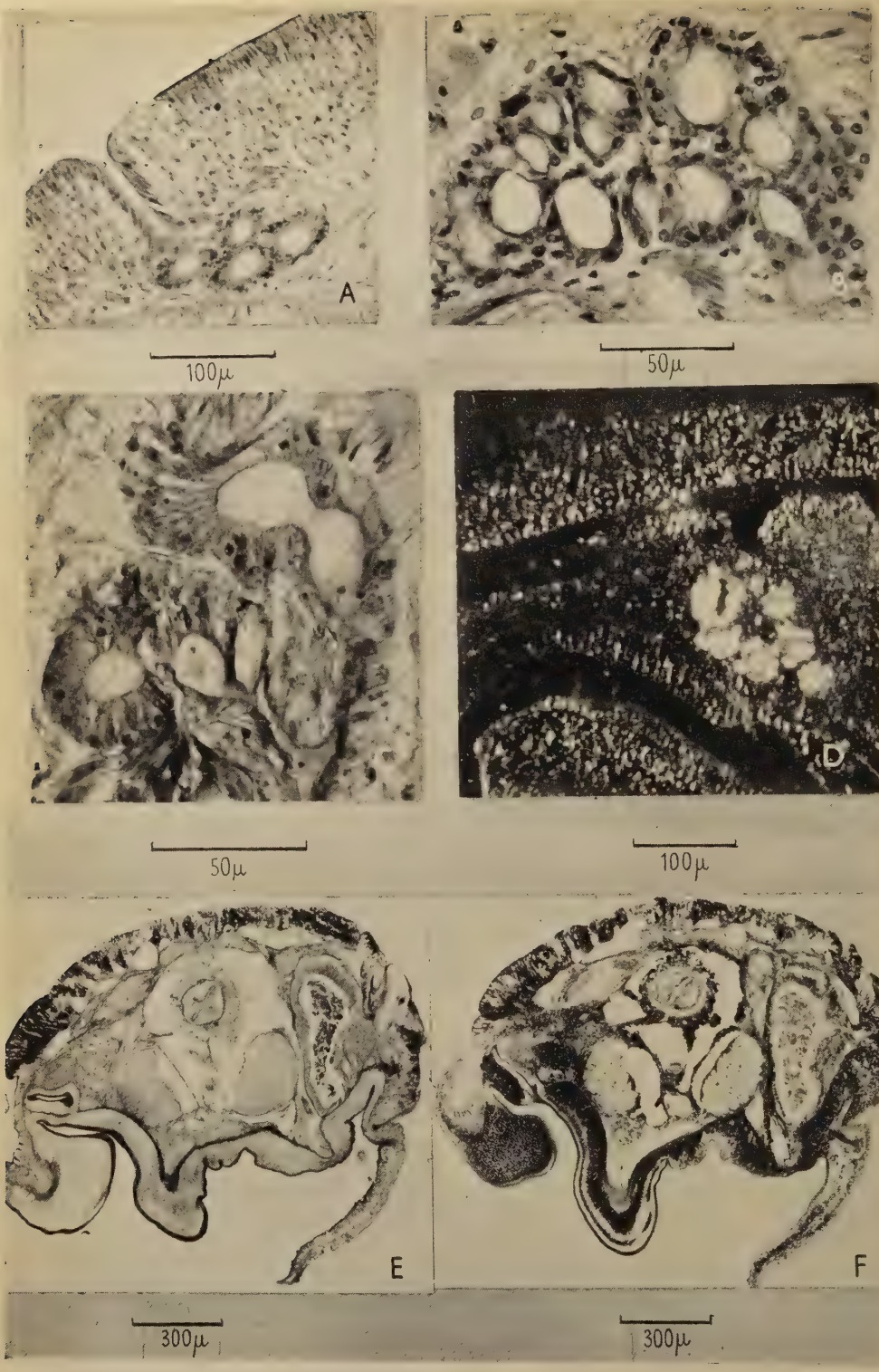

FIG. 2

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elongate columnar (fig. 2, A) and are always filled with mucigen. The mucous secretion forms part of the organic component of the tube and is mixed with the calcareous secretion of the tubulo-racemose glands by a rippling movement of the peristomial collar.

This term mucus, or mucin, has no precise chemical significance, the chemical composition varying with the source of it, and any particular mucus may contain one of several glycoproteins. In the following account the nomenclature used is based on the classification of the mucopolysaccharides by Meyer (1945).

Southgate's mucicarmine stains the epithelial mucus-cells bright red. Other parts that are stained, but less intensely, are the mucus-cells of the oesophagus, the lining of the tubules of the calcium-secreting glands, and to a less extent the basement membrane of the dorsal epidermis and the external cuticle. Similar results were obtained after staining in Mayer's muchaematein. After staining sections with a 0·5% aqueous solution of basic fuchsin, most tissues stained red. By varying the pH of the staining solution with HCl it was possible to stain the mucigen of the ventro-lateral epithelium selectively. A basic fuchsin solution (pH1) will stain the mucigen red, leaving the rest of the tissues unstained. The results of staining with these three solutions indicate nothing more than the location of the mucigen, and that it has an affinity for basic dyes which is retained in solutions with low hydrogen iron concentration.

After staining with toluidine blue, the mucigen of the ventro-lateral epithelium, the oesophageal mucus-cells, the basement membrane, and general connective tissues were metachromatic red or pink. This result is considered to indicate the presence of sulphomucopolysaccharides (Lison, 1936; Landsmeer, 1951).

Before the procedure of the PAS technique the sections were incubated in diastase for 20 minutes. There is a considerable amount of glycogen in the tissues of *Pomatoceros* (fig. 2, E and F). After removal of the glycogen the PAS method gives a positive result with the mucigen of the ventral and lateral epithelium, the lining of the oesophageal lumen, the basement membrane, and the cuticle (fig. 2, E). Sections treated with an acetylating mixture, which blocks the hydroxyl groups (McManus and Cason, 1950), and then treated with PAS, were completely unreactive. The mucigen in the ventral and lateral epithelium, therefore, contains *a*-glycol groups (-HCOH-HCOH-). Such a characteristic is not the property of all mucopolysaccharides. For example, chondroitin sulphuric acid and heparin trisulphuric acid do not react (Jorpes,

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FIG. 2 (plate). A, a transverse section through the calcium-secreting gland, showing the major duct to the exterior. 6 μ. Hansen's trioxyhaematein and orange G.

B, a transverse section of a calcium-secreting gland, showing distortion after Bouin fixation. 6 μ. Heidenhain's iron haematoxylin and orange G.

C, a section of a calcium-secreting gland, showing junctions of the duct system. Fixation by Lewitsky's fluid. 6 μ. Heidenhain's iron haematoxylin without a counterstain.

D, a section of a gland after micro-incineration, showing heavy deposits of calcium ash in the tubes and cells. 8 μ.

E, a transverse section through the peristomium after PAS with the glycogen removed. 8 μ.

F, a transverse section of the peristomium after PAS without the removal of glycogen. 8 μ.

Werner, and Aberg, 1948), whilst a positive result is given by hyaluronic acid and heparin monosulphuric acid. Hotchkiss (1948) states that the amount of reagent fixed after PAS is dependent on the actual weight of *a*-glycol groups present. Comparison of the peristomial tissues after the PAS procedure shows that the concentration of the red colour in the mucigen of the ventral and lateral epithelium is significantly less than that in the basement membrane. Another type of mucus, the mucigen in the goblet cells of frog's stomach, reacted to produce a very intense red compared with the moderate red of the mucigen in the epithelium of *Pomatoceros*.

Histochemical demonstration of the uptake of ferric iron by the mucigen in the peristomium. Hale (1946) suggested a method for the demonstration of acid 'polysaccharides', particularly hyaluronic acid, in tissue sections. After application of the procedure it was not found to be specific for such substances, although the mucigen of the ventral and lateral epithelium of the peristomium gave a strikingly stronger Prussian blue colour than the other tissues. Control sections when treated with potassium ferrocyanide (2% solution) and potassium ferricyanide (2%), without prior immersion in the iron solution, failed to react. Sections which were incubated at 37° C with hyaluronidase (Benger product, in frog Ringer solution) showed no difference in intensity of the Prussian blue colour from that of a normally treated section.

After increasing the acidity of the acetic iron solution (up to 50% CH_3COOH), there was no visible difference in iron uptake of the mucigen of the ventro-lateral epithelium. There was, however, a reduction in colour of the Prussian blue in the other tissues. Hydrochloric acid was used in place of acetic acid to give lower pH to the ferric iron solution. In solutions of acid concentration 1·5 N, only the basement membrane and the mucigen gave a Prussian blue reaction. Uptake of iron by the basement membrane does not take place in more acid solutions. In iron solutions (5 N and 6·5 N HCl strength), certain parts of the mucigen are unreactive. The mucigen which retains the affinity for iron under these conditions is that found at the exterior end of the mucus-cell. This affinity for iron under these conditions is not specific for the mucigen of the epithelium in the peristomium. Other epidermal mucus-cells of the thorax and abdomen react similarly.

The presence of iron taken up from iron solutions can be shown by micro-incineration. Iron when oxidized and in the form of ash is yellow to deep red (Scott, 1934). This is easily visible and incinerated sections of the peristomium reveal the heaviest ash deposits of orange ash in those areas where there is the strongest Prussian blue colour.

Another method of detecting the iron uptake by tissue sections is as the sulphide (Wigglesworth, 1952). Slide preparations referred to earlier have been repeated, with the use of ammonium sulphide to detect the iron. Similar results were obtained to those with Prussian blue as the end-product.

Wigglesworth (1952) suggests that the carboxyl and phosphoric acid groups of tissue constituents are responsible for the uptake of iron. In addition Wigglesworth considers the uptake of iron, and the demonstration of it histo-

chemically, to be a test for the presence of such free acidic groups. The mucigen of the ventral and lateral epithelium combines with ferric iron in acid solutions when the rest of the tissues cease to react. This indicates that there are perhaps acidic groups in the mucigen other than carboxyl or phosphoric acid groups which remain ionized in these acid-iron solutions of low pH. The electrolytic dissociation of three acidic groups in biocolloids, carboxyl (-COOH), phosphate ($-OPO_3H$), and sulphate ($-OSO_3H$), is determined in part by the pH of the medium (Overbeek and Bungenberg de Jong, 1949). Carboxyl groups have a low dissociation constant and will lose their negative charge in acid medium before the phosphoric acid groups. Sulphuric acid ester groups, being much more acidic, will remain ionized in much stronger acid solutions. It is possible that the iron in the mucigen is linked in some way with the sulphuric acid groups present in the carbohydrate part of the glycoprotein.

Experiments with heparin, which can be obtained in a pure state, support this view. Heparin, a sulphomucopolysaccharide (Meyer, 1945), is that substance which is thought to be in the mast-cells of rat connective tissues and which gives a metachromatic red after staining with toluidine blue (Holmgren and Wilander, 1937). Heparin is water-soluble, does not give any protein reactions, and is not precipitated by acids. It is, however, precipitated by basic lead acetate (Howell, 1924). Pure heparin was precipitated by a lead acetate-formalin mixture from a strong aqueous solution. This precipitate was dried in an oven and then embedded in a thin layer of albumen on a glass slide. This preparation was placed in absolute alcohol for 1 minute and then used for testing. With toluidine blue the heparin precipitate stained red and this contrasted with the albumen, which was lightly stained blue. With PAS the heparin gave a pale pink reaction. This weak result indicates the low concentration of free *a*-glycol groupings and this may be because heparin normally occurs as a mixture of heparin mono-, di-, and trisulphuric acids (Jorpes and Sven, 1948). The heparin trisulphuric acid will not react with PAS. After treatment by the iron method, with an acid ferric chloride solution containing 2·5 N HCl, the albumen remained uncoloured and the heparin particles were blue. Heparin, therefore, has an affinity for iron similar to that of the mucigen of the ventro-lateral epithelium of the peristomium of *Pomatoceros*. Now heparin does not contain phosphoric acid groups and combines with iron in acid solutions too strong for the ionization of carboxyl groups.

In the case of the mucigen and the heparin, where the effect of carboxyl and phosphate groups can be disregarded, it appears that the sulphuric acid ester groups of the carbohydrate components are linking in some way with the ferric iron.

Discussion

The recognition of organs in *Mercierella* (Swann, 1950) and *Pomatoceros* which are responsible for the production of a calcareous material solves only one of many problems in the study of tube formation.

The inorganic component of Serpulid tubes is mainly calcium carbonate

but it is incompletely known to what extent other calcium salts or other metals are incorporated (Vinogradov, 1953). Potts (see Robertson and Pantin, 1938) considered that the calcium carbonate of *Pomatoceros* tubes exists in the form of aragonite and confirmation of this was obtained during the course of the present work. The peristomial calcium-secreting glands are the source of the major part of the inorganic material, but a secondary source is the mucigen of the ventro-lateral epithelium which contains a relatively large amount of calcium.

There are at least two sources of the organic component of a *Pomatoceros* tube. The major contributor is the ventro-lateral epithelium in the peristomium, and the second is the organic matrix which is present in the tubules of the calcium-secreting glands. The mucigen of the ventro-lateral epithelium is a mucous substance, which contains a sulphomucopolysaccharide. Once this is secreted, changes may occur as it passes into the sea-water medium. Cartilage is known to have an equivalent capacity for the cations sodium, calcium, and barium (Boyd and Neuman, 1951). The same authors consider that chondroitin sulphuric acid is the principal binding agent in cartilage and they note the close correlation between this binding capacity and the sulphate content. The mucigen in the ventro-lateral epithelium, which is chemically similar to the chondroitin sulphuric acid of cartilage in that both contain sulphomucopolysaccharides, may react with and bind cations once it is secreted into the fold of the peristomial collar. It is this secreted mucous substance which is mixed with the secretion of the calcium-producing glands to form the tube.

The tube-forming material present in the fold of the peristomial collar is moulded by the collar on to the anterior end of the tube. A requisite of this moulding process is that the calcareous material should remain in a pliable state during deposition and then harden to form part of the tube.

I wish to thank Professor A. D. Hobson and Mr. J. Shaw for their encouragement and advice during the progress and presentation of this work, which was carried out during the period of tenure of a grant from the Department of Scientific and Industrial Research (1950–3).

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Studies of Serpulid Tube Formation

II. The Calcium-secreting Glands in the Peristomium of *Spirorbis*, *Hydroides*, and *Serpula*

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With 2 plates (figs. 1 and 2)

Summary

Descriptions of the calcium-secreting glands, which produce the calcareous component of the tubes, are given for *Spirorbis*, *Hydroides*, *and Serpula*. In *Hydroides* the gland is a simple tubule. The gland in *Spirorbis* is tubular but the gland-cells have unusual extensions which project into the lumen. In the centre of these extensions there is always a strongly basiphil substance. In *Serpula* the glands are tubulo-racemose or compound tubular, similar to those in *Pomatoceros*.

In addition to the calcium-secreting glands in *Serpula* there are two ventral calcium sacs in the posterior part of the peristomium which secrete calcareous material. The sac wall has a peculiar structure, being non-cellular and consisting of two layers, one basiphil the other acidophil. In the acidophil part two layers are seen, one fibrous and the other optically homogenous in which numerous small bodies are found.

Introduction

SWANN (1950) figured and described major subcollar glands in *Mercierella enigmatica* Fauvel. These are the peristomial calcium-secreting glands which produce the calcareous component of the tube. They are simple saccular glands and quite different from the tubulo-racemose glands in *Pomatoceros* (Hedley, 1956). Major subcollar glands have been recognized in *Serpula vermicularis* L. but they have not been described or figured (Swann, 1950). Sac-shaped organs in the peristomium of *Marifugia cavatica* (Absolon and Hrabě, 1930) are in a position similar to the calcium-secreting glands of other serpulids and are most likely the glands responsible for calcium production. Zur Loye (1908) gives an account of glands in the peristomium of *Spirorbis* which are certainly those recognized in this account as the calcium-secreting glands.

For reasons to be given later the name major subcollar gland, which has been proposed for the peristomial organs producing calcium carbonate, is not adopted and the name calcium-secreting gland is used.

The ventral shield referred to in this account is an area on each side of the peristomium in *Hydroides norvegicus* Gunnerus, *Spirorbis borealis* Daudin, and *Serpula vermicularis* L., which is in a position comparable with the ventro-lateral mucous areas in the peristomium of *Pomatoceros* (Hedley, 1956).

This paper deals with the calcium-secreting glands of three serpulids and demonstrates the wide range of form found in the Serpulidae of these organs, which produce calcareous material for tube formation.

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Materials and Methods

Specimens of *Hydroides* and *Serpula* were obtained from the Marine Biological Association, Plymouth. *Spirorbis* was collected from the intertidal zone at Cullercoats, and preserved specimens of *Mercierella* were obtained from the British Museum (Natural History).

All micro-anatomical detail was obtained from worms fixed in Lewitsky's fluid (Flemming-without-acetic), Zenker's, or Helly's. For *Hydroides* 4 hours' fixation was adequate; 2 hours' was sufficient for *Spirorbis*, and 6 hours' for *Serpula*.

Examination of worms with reflected light after they had been fixed, dehydrated, cleared in methyl benzoate / celloidin, and washed in benzene provided a useful guide to the presence of calcium in the calcium-secreting glands.

Spirorbis borealis Daudin

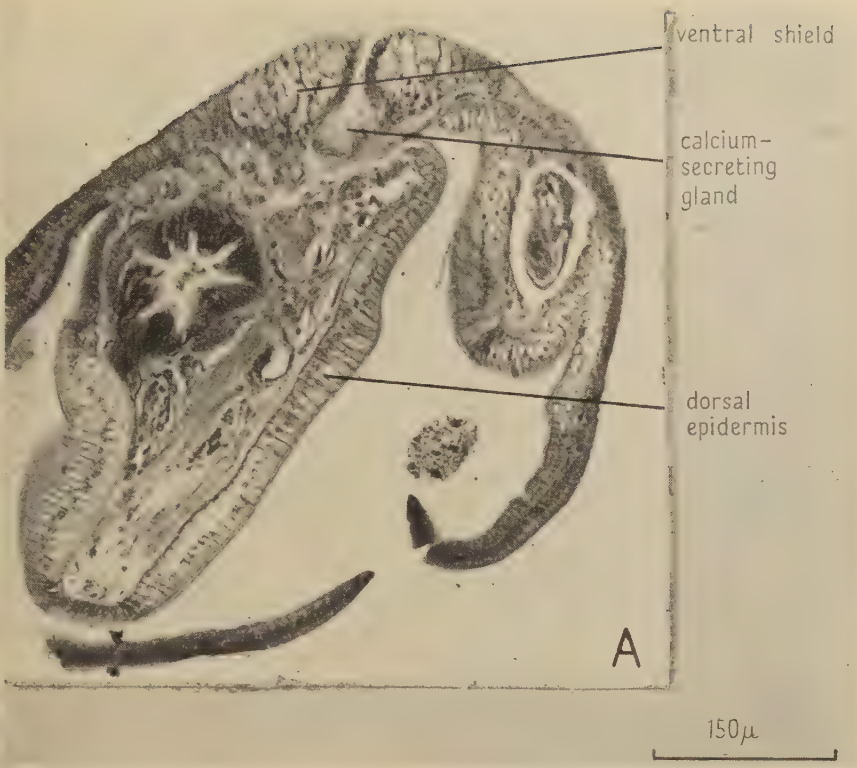
A fine white rod is seen in the middle of each ventral shield area when worms which have been fixed and cleared are observed with reflected light. This white substance is the calcareous content of the calcium-secreting glands and is dissolved in HCl.

The calcium-secreting glands are tubular and cytologically quite different from those found in the other serpulids examined. In worms about 2 mm long the average size of the glands is 90μ by 40μ. The duct to the exterior passes through the ventro-lateral epithelium (fig. 1, A) and posterior to this point is the gland mass in the subepithelial connective tissue. Muscle-fibres are present in the connective tissue immediately surrounding the gland. Numerous structures extend from the gland epithelium layer into the lumen (fig. 1, B). The appearance of these varies with the fixative used and their great distortion after acid fixation makes interpretation difficult. A clearer picture is obtained after fixation in a neutral formalin / absolute alcohol mixture, which does not dissolve the calcium content. In such a preparation the projecting parts appear as extensions of the cells of the gland. In each extension there is a central basiphil club-shaped component (fig. 1, B), the thin end of which terminates at the level of the beginning of the projection of the cell. This arrangement of the gland-cells and their extensions into the lumen is typical of the whole gland with the exception of the region of the duct to the exterior. Here the cell extensions are absent although in some glands they may be present as small swellings.

In material fixed in a neutral solution the cell extensions are full of small calcium granules, but no calcium was detected in the basal part of the cells.

Fig. 1 (plate). A, an oblique transverse section of the peristomium of *Spirorbis*. 7 μ. Fixation in neutral alcohol / formalin. Hansen's trioxyhaematein and orange G.

B, transverse section through calcium-secreting gland of *Spirorbis*. 7 μ. Fixation in neutral alcohol / formalin. Hansen's trioxyhaematein and orange G.



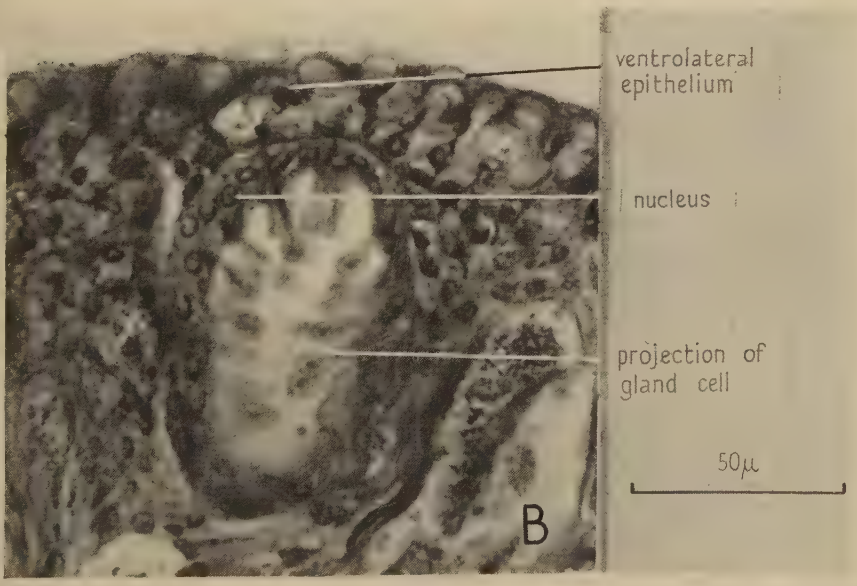


FIG. 1

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Hydroides norvegica Gunnerus

In worms examined alive a white pigment patch, resistant to mineral acid, is seen on the lateral edge of the ventral shield. In worms fixed in a neutral solution and cleared and examined with reflected light, a single white rod is seen in each side of the peristomium in addition to the white pigment patch. The white rod begins in a depression in the middle of the ventral shield and ends posteriorly. This white rod dissolves in HCl and is the calcium content of the calcium-secreting gland. The white pigment area consists of a number of cells of the ventro-lateral epithelium, which is full of granules. They are not calcareous as no white ash deposit remains after incineration. The cells stain jet black in Heidenhain's iron haematoxylin and this colour is retained much longer than by any other tissue during differentiation in iron alum (fig. 2, A).

The calcium-secreting glands are simple tubular glands (fig. 2, A). Each opens to the exterior in a depression in the ventral shield. From this point the tubule passes through the epithelial layer into the connective tissue and extends posteriorly. In all worms which have been examined the glands have consisted of a single tubule with the secreting cells radially arranged round a central lumen. Muscle-fibres are present in the connective tissue immediately surrounding the gland. The length of the glands in an average size worm (15 mm long) is 200 to 250 μ and the diameter 40 to 50 μ.

Serpula vermicularis L.

When the living worm is removed from its tube two conspicuous white areas are seen, one on each side of the mid-ventral line in the posterior region of the peristomium. Immediately a thick white substance pours out from each of these two organs (seen in section fig. 2, B). After about 15 seconds all the white contents have been ejected from the worm and the white areas are no longer visible. These organs are to be considered and are referred to as the ventral calcium sacs. They are not to be confused with the calcium secreting glands.

Worms which have been removed from their tubes and kept in sea water produce calcareous deposits in the collar fold. These deposits appear initially on the surface of the ventral shield and are still produced by worms in which the ventral calcium sacs have been cauterized. In worms fixed in neutral solution, cleared, and viewed with reflected light, a number of small white rods are seen in the middle of the ventral shield. These disappear in HCl and are the calcium contents of the calcium-secreting glands. In addition to these white rods a white pigment patch is present in the lateral edge of the ventral shield. This is a constant external feature and similar to that seen in *Hydroides*.

The calcium-secreting glands. These are tubulo-racemose and each opens by a single duct to the exterior in the middle of the ventral shield. This duct passes through the ventro-lateral epithelium and is the most anterior part of the gland. The remainder of the gland occupies an area posterior to this opening duct. This is different from the arrangement in *Pomatoceros* where the

opening duct comes from the central part of the gland and never from one end. In *Serpula* most glands are compact but in some a single tubule extends much farther posteriorly than the others. The size of a gland in an average worm (2 cm long) is approximately 200 μ (anterior-posterior face) and 300 μ (ventral-dorsal face). A single tubule is approximately 40 to 50 μ in diameter and similar both in size and structure to a tubule in a gland in *Pomatoceros*, and to the single tubule, which is the gland, in *Hydroides*.

The white pigment area. This is seen on the lateral edge of the ventral shield and is composed of epithelial cells full of granules. These are resistant to dilute mineral acids and acid fixatives. The pigment areas are noted because of their near proximity to the calcium-secreting glands and because of the confusion which has arisen and may arise from external examination alone. The areas are constant external characters found in all examined specimens of *Hydroides* and *Serpula*. In addition their contents are non-calcareous and do not contribute towards the inorganic component of the tube.

The *ventral calcium sacs* (figs. 2, B, and 3). Because the contents of these sacs are ejected immediately worms are removed from their tubes, and because the sacs are found empty after worms have been fixed in their tubes, it is necessary to narcotize the specimens when a sample of the sac contents is required. After 8 hours in equal volumes of sea water and 7·5% magnesium chloride the worms can be taken from their tubes without a concomitant liberation of the sac contents. Samples from the sacs were obtained in a fine capillary and transferred to a slide. The material will dissolve in HCl. After incineration to 600° C a dense white ash remained, to which was added a drop of dilute HCl and dilute H_2SO_4. Characteristic crystals of calcium sulphate were obtained.

The anterior surfaces of these organs are approximately 250 μ posterior to the posterior end of the tubulo-racemose calcium-secreting glands. They are in the posterior part of the peristomium, always on each side of the mid-ventral line and surrounded by the connective tissue between the ventral epithelium and the oesophagus. On the lateral side of each sac there is the ventral longitudinal muscle.

Each sac has a single duct passing through the ventral epithelium to the exterior. The maximum diameter of these ducts in preserved material is 10 μ, but observations of the living worm indicate that this opening is much larger when the contents are being ejected. Each sac is surrounded by connective tissue except in the region of the opening duct, and has no direct connexion with any other peristomial organ. It is concluded that the calcareous contents are secreted by the sac wall.

The sac wall varies in thickness throughout the length of the sac. During fixation this wall may come away from the connective tissue and be distorted.

FIG. 2 (plate). A, an oblique transverse section of the peristomium of *Hydroides*. 5 μ. Fixation in Zenker-acetic. Heidenhain's iron haematoxylin and orange G.

B, transverse section of the peristomium of *Serpula*, posterior to the calcium-secreting glands and showing the ventral calcium sacs. 6 μ. Fixation Bouin. Haemalum, and eosin.

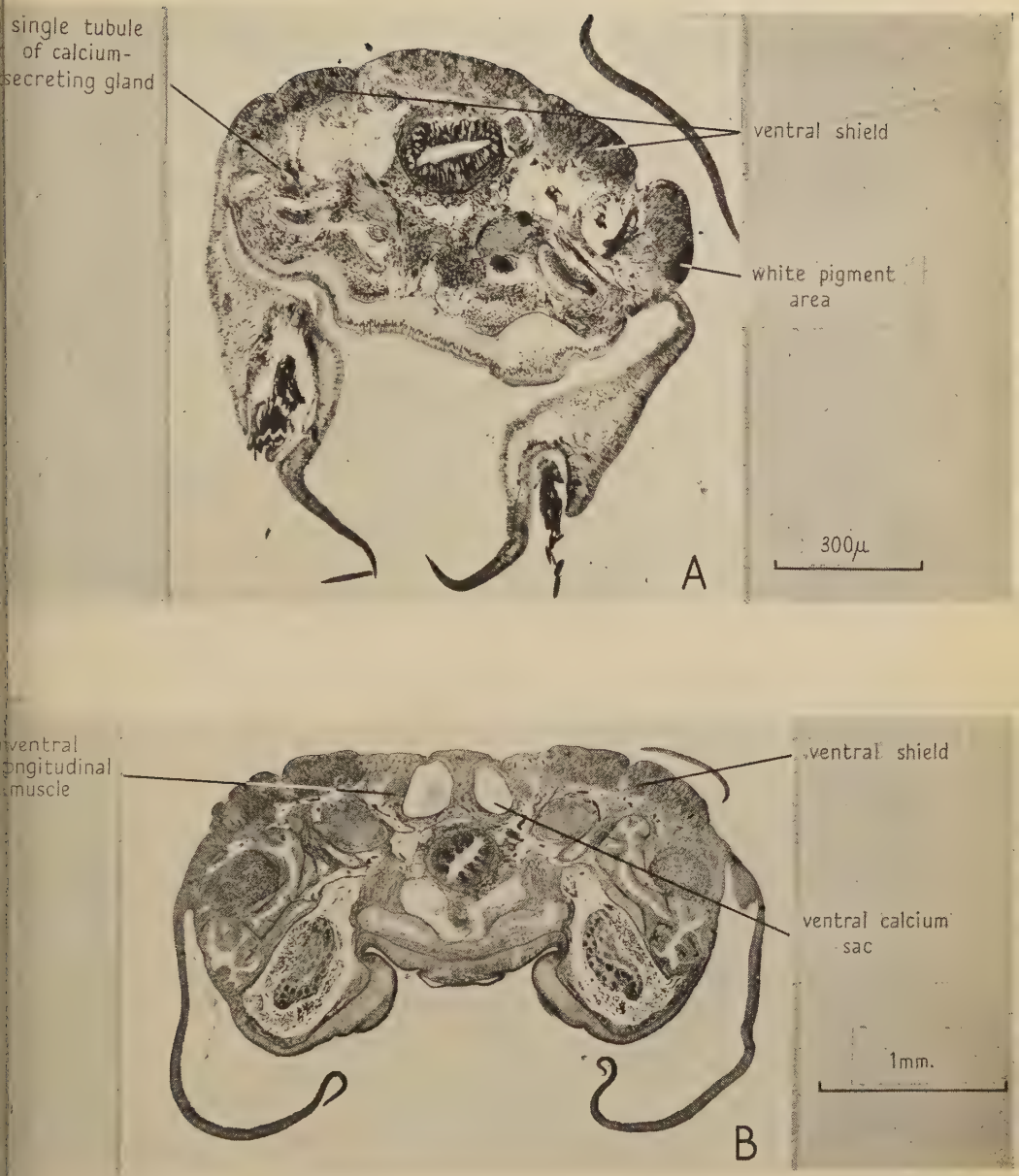

FIG. 2

R. H. HEDLEY

In addition to distortion of this type the sac wall is shrivelled and vacuolated after alcohol fixation and vacuolated after a mercuric chloride / formalin fixative. In the lumen of the sac there is usually some organic matrix remaining which is basiphil and stains pink after toluidine blue. Bordering the lumen is a very thin layer of the sac wall (fig. 3) which is basiphil and stains blue after toluidine blue. The rest of the sac wall is acidophil and does not stain at all with toluidine blue (fig. 3). This acidophil layer contains two structurally different regions. The one adjacent to the basiphil layer consists of a fibrous substance whilst the other region next to the connective tissue appears homogenous. In this latter region there are numerous small spherical bodies which have a surrounding halo (fig. 3). These are present after all fixatives, they are

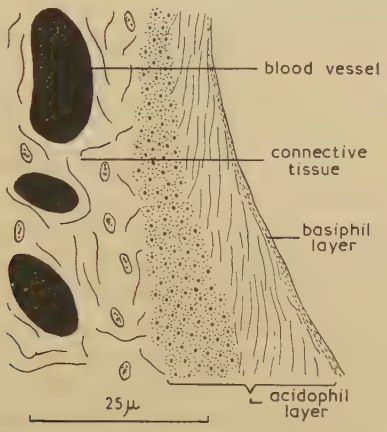

Fig. 3. Diagram of a section through part of the wall of the ventral calcium sac of *Serpula*

unstained by toluidine blue, appear orange after eosin when the surrounding optically homogenous tissue is red, and stain black after Heidenhain's iron haematoxylin. The connective tissue surrounding the sac wall is structurally similar to the connective tissue elsewhere. After staining with toluidine blue, however, the connective tissue immediately surrounding the sac wall is stained an intense red compared with the pink colour of the connective tissue in other parts of the section.

This sac wall has a most peculiar structure. It is not a glandular epithelium and is in no way a cellular structure. Nevertheless, this tissue and perhaps the connective tissue surrounding it is responsible for the production of the calcareous and organic contents of the ventral calcium sacs. It is possible that the connective tissue surrounding it has a peculiar role in either producing the sac wall, or contributing to the production and secretion of the sac contents, or both.

Discussion

It is established that in the serpulids *Mercierella* (Swann, 1950), *Pomatoceros* (Hedley, 1956), *Spirorbis*, *Hydroides*, and *Serpula* there are two exocrine calcium-secreting glands in the peristomium, the collar-bearing segment.

Usually these glands are situated immediately inside the ventro-lateral epithelium; nevertheless, variation in position does occur both between species and within a species. For example, in *Pomatoceros*, *Hydroides*, and *Serpula* the calcium-secreting glands may extend into the base of the collar, whilst in *Mercierella* (those specimens the author has personally examined) and *Spirorbis* the glands have always been found posterior to the level of the collar attachment. A correlation exists between the position of the glands and the ventral shield areas, which are mucus-secreting areas and the major contributor of the organic part of serpulid tubes (Hedley, 1956). The calcium-secreting glands always open to the exterior in the middle of these ventral shields. In those cases where the glands extend into the base of the collar the associated ventral shield areas are also found relatively more anterior. Consequently, when considering the question of position it is useful to consider a unit composed of the ventral shield and the gland. The position of the unit varies among individuals and species within the anterior, ventrolateral region of the peristomium.

The name major subcollar gland has been proposed for the glands which are responsible for the secretion of calcareous tube material in serpulids (Swann, 1950). This name has not been adopted because of the confusion which may arise over the adjectives major and subcollar. Compared with other serpulids there is a unique arrangement in the peristomium of *Serpula*. The tubulo-racemose glands are comparable with those in *Pomatoceros*. In addition to these glands in *Serpula* there are the ventral calcium sacs, also in the peristomium. These contain calcareous material which passes to the exterior through the sac duct and it is considered here that they contribute towards the tube construction. Because of this the term major used to distinguish the calcium-secreting glands from the unicellular epidermal glands will lead to confusion. This will be so especially in *Serpula* and possibly in other serpulids where four peristomial organs may be present, all containing and secreting calcareous material.

A great structural variation of the calcium-secreting glands in the different serpulids has been demonstrated. These range from the simple saccular organs in *Mercierella* and perhaps *Marifugia* to the simple tubular form in *Hydroides* and the tubulo-racemose or compound tubular types in *Pomatoceros* and *Serpula*. Furthermore, in *Spirorbis* the glands, although tubular, have an unusual glandular epithelium from which extensions of the cell project into the lumen. Considering this range of form we might expect to find other types in other genera of the Serpulidae. A study of these peristomial glands in *Filograna implexa* Berkeley and in *Salmacina dysteri* (Claparède) may clarify the suspected relationship of these two serpulids. McIntosh (1923) and Faulkner (1930) contend there is insufficient difference between the two to warrant their separation into genera, whilst Fauvel (1927) chooses to keep the two separate. If the calcium-secreting glands prove to be similar in both serpulids it would add support to the view favouring synonymy, whilst two different gland types would suggest the retention of Fauvel's classification, especially when it is

noted that the gland form of those serpulids reported here shows consistency within a species.

I wish to thank Professor A. D. Hobson for his help and encouragement during the course of this work; also Mr. J. Shaw for his advice and interest. I am grateful to the Director of the Marine Laboratory, Plymouth, for the facilities offered during my occupation of the University of Durham table in July 1952. I also wish to thank the Trustees of the British Museum (Natural History) for preserved specimens of *Mercierella*. This paper is an account of part of the work carried out during the period of tenure of a grant from the Department of Scientific and Industrial Research (1950–3).

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The Antennal Pulsating Organs of Mosquitoes and other Diptera

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Summary

The antennal pulsating organ of mosquitoes consists of two vesicles, each sending a blood-vessel to the antenna of its side and each connected to the aorta by a muscle. Contraction of the muscle causes blood to enter the vesicle through a valve and relaxation allows an elastic contraction of the vesicle so that the blood is discharged through the blood-vessel to the antenna. The organ appears to be myogenic.

Antennal pulsating organs occur in most families of the Diptera Nematocera but not all, and a similar organ is present in *Drosophila*.

Introduction

THE heads of certain Diptera contain a small organ which has long been familiar to the morphologists who have studied these insects but which has generally received only brief mention incidental to other observations. Superficially the organ has the appearance of a pair of vesicles lying under somewhat modified areas of cuticle, each vesicle sending a prominent nerve to the brain. Dufour (1851) described the organ in *Tipula oleracea* and suggested that it represented vestigial ocelli. A similar nature has been ascribed to the organ in *Chironomus* (Miall and Hammond, 1900), *Culex* and *Anopheles* (Thomson, 1905), and *Phlebotomus* (Christophers, Shortt, and Barraud, 1926). Jobling (1928), studying *Culicoides*, considered the organ to be functional ocelli. Recently it has been described at some length in *Aëdes aegypti* and has been called a sense organ, possibly sensitive to changes in atmospheric pressure (Day, 1955).

The ocellar nature of the organ was questioned by Clements (1953), who showed that the structures which had been called nerves were muscles running to the aorta between the lobes of the brain. In fact, clues to its real function were provided by Eggers (1924) and Risler (1953, 1955), who described blood-vessels entering the antennae of mosquitoes, although they did not describe their origin from the organ in question here. Miller (1950) briefly mentioned a single vesicle in the head of *Drosophila* from which two muscles ran to join the aorta at the back of the head. He suggested that this organ resembled the antennal pulsating organs of other insects but was unable to show how it could function as such. In the pages which follow, a description is given of the structure of the organ in mosquitoes, with an account of its probable method of functioning. These views were briefly expressed by Clements (1956).

Methods

The use of a polarizing microscope was indispensable for studying the distribution of muscle fibres in the organ and for this purpose unstained sections

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were used. In addition, sections were stained with Masson's trichrome stain after fixation in alcoholic Bouin, or with Samuel's silver stain after fixation in the recommended modification of Bouin's solution (Samuel, 1953).

Structure and Function in *Culex pipiens* L.

The structure of the organ can be seen best in sagittal section. It consists of two vesicles lying medially, just below the compound eyes, each giving off a vessel to the antenna of its side and each connected by a muscle to the aorta (figs. 1, 2). As Thomson (1905) has shown, the organ first appears during the

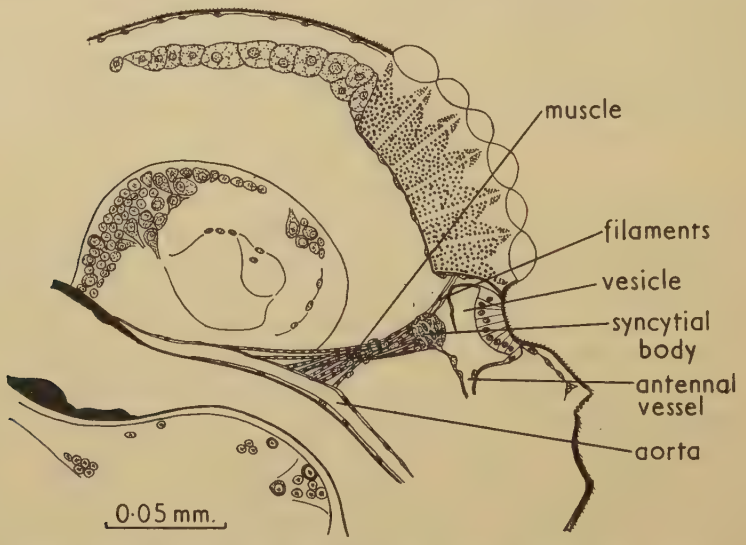

Fig. 1. Sagittal section through the head of *Culex pipiens*, showing the antennal pulsating organ.

pupal stage. It is not present immediately after the pupal moult but appears during the following 24 hours.

The organ shows externally as a pair of depressions, each about the width of an ommatidial facet, situated just under the compound eyes, one on each side of the midline. In section, the cuticle of the depression is seen to be flat, of normal thickness and bearing minute hairs. The epidermis underlying the cuticle forms the distal end of the vesicle and this small group of cells has the nature of columnar epithelium. The cells are very large with their nuclei disposed towards the free border. The walls of the vesicle arise from the edges of the group of columnar cells. The walls are clearly cellular near the cuticle but posteriorly form a flattened, nucleated layer. An opening into the vesicle is always present posteriorly, the dorsal wall extending into the sac and apparently forming a valve which would close the opening under certain circumstances (figs. 1, 2).

Ventrally, the sac gives rise to a vessel which runs into the antenna of the same side. This vessel has been called a trachea (Clements, 1953; Day, 1955),

but it is more likely to be the blood-vessel described as entering the antenna by Eggers (1924). The wall of the vessel is thick and nucleated and it does not show the taenidia of tracheae of the same size. Occasionally, sections show branches from the trachea to the antenna which rise vertically to pass close to the vesicle but do not penetrate it.

Behind the vesicle is a spherical mass consisting of a very large number of nuclei narrowly separated by cytoplasm, apparently without cell boundaries. These are called receptor cells by Day (1955), but they bear no resemblance to typical sense cells and apparently are not innervated. From this syncytial

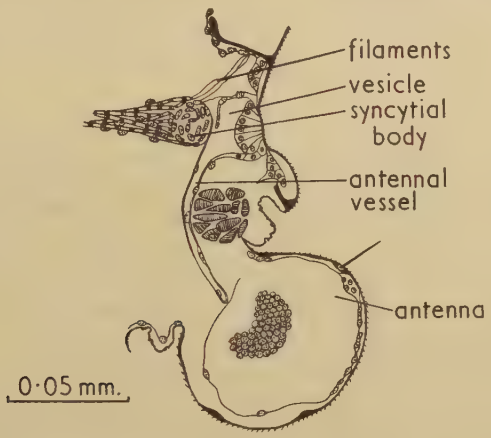

Fig. 2. Sagittal section through the antennal pulsating organ and antenna of *Culex pipiens*.

body fine filaments run to the vesicle and to the integument, probably serving to hold the vesicle in position during contraction of the muscle. Possibly the syncytial body has the nature of connective tissue.

The aorta of mosquitoes is said by Jones (1954) to end as an inverted trough between the supraoesophageal ganglion and the pharynx, but in longitudinal sections it can be seen to extend as a tube over the pharynx for some distance in front of the brain. Between the syncytial body and the aorta run a number of distinctly striated muscle fibres formed into a rather loose bundle without a sheath. The fibres appear to splay out over the syncytial body and they are possibly attached to the vesicle. Some of the fibres are attached to the aorta in front of the brain; others, as can be seen with polarized light, run under the brain to be attached to the aorta much farther back. It is these two muscle bundles which have been called nerves by previous authors. The muscles show strong birefringence with polarized light. The vesicle, antennal vessel, and aorta are weakly birefringent; no muscle fibres can be seen in them, so they may consist of an elastic connective tissue.

Despite examination of a large number of specimens, including silver-stained material, no nervous connexions to the pulsating organ have been found. Jones (1954) concluded that the mosquito heart was myogenic, after failing

to find any direct innervation and after applying certain physiological tests. It appears that the pulsating organ is also myogenic.

Contraction of the muscles of the pulsating organ will extend the vesicles and will also, as occasional sections show, enlarge the lumen of the aorta in front of and below the brain. It may be presumed that blood will enter the expanding vesicles through the valves and that relaxation of the muscles will allow an elastic contraction of the vesicles causing a flow of blood through the antennal vessels, the valves being closed, and also forward from the aorta. Without experimental evidence it is impossible to say what part the pulsating organ plays in the movements of the antennal flagella, but if the pulsating organ is not under nervous control it cannot effectively control flagellar movement.

Other Diptera

An antennal pulsating organ is present in several families of the Diptera Nematocera. In the Culicidae it has been found in the genera *Anopheles*, *Culex*, *Theobaldia*, *Aëdes*, and *Chaoborus*. It has been recorded in Tipulidae (Dufour, 1851), Chironomidae (Miall and Hammond, 1900), Ceratopogonidae (Jobling, 1928), Psychodidae (Christophers, 1926), and Rhyphidae, Cecidomyiidae Mycetophilidae, and Sciaridae (Day, 1955). A study of the organ throughout the order, now being undertaken by the present author, has shown the organ to be present also in the Ptychopteridae (*Ptychoptera contaminata* (L.)), Trichoceridae (*Trichocera saltator* (Harris)), Scatopsidae (*Scatopse transversalis* Loew) and Blepharoceridae (*Liponeura* sp.). The organ is not to be found in either sex in *Thaumalea testacea* Ruthe (Thaumaleidae), *Simulium ornatum* Mg. (Simuliidae), or *Dilophus febrilis* (L.) (Bibionidae).

The structure of the organ is broadly similar in mosquitoes and the other families of the Diptera Nematocera, but minor differences are to be found. For example, the cuticle over the organ is often raised slightly rather than depressed, and in *Chironomus* the pulsating organ lies within the frontal tubercles. The epidermis is rarely hypertrophied, although this condition occurs in *Trichocera saltator* as in mosquitoes. As Day (1955) has pointed out, the presence of the antennal pulsating organ together with ocelli in certain families shows that these two organs are quite distinct.

Day was unable to find the organ in *Neoexaireta spinigera* Wied. (Stratiomyidae) or *Musca domestica* L., and considered that it was probably entirely absent from the Diptera Brachycera. However, Miller's account of an antennal pulsating organ in *Drosophila melanogaster* Mg. has been confirmed by examining *D. funebris* (Fab.), where it consists of a single vesicle, below the ptilinum, from which two muscles arise directly without a distinct syncytial organ and run to the aorta. Antennal vessels pass from each side of the vesicle to the antennae.

Examination of *Panorpa germanica* L. (Mecoptera) has revealed an antennal pulsating organ strikingly similar to that found in Diptera. Accessory hearts supplying the antennae have been described in Orthoptera, Dictyoptera, and

Hymenoptera (Wigglesworth, 1950) and it seems likely that they will be found in most insects.

Certain of the observations included in this paper were first made by Sir Rickard Christophers, F.R.S., whose continuous interest has been greatly appreciated. It is a pleasure to express my thanks also to Dr. S. M. McGee-Russell and Mr. B. Jobling for the help which they have given.

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The Growth and Development of a Viviparous Compound Ascidian, *Hypsistozoa fasmeriana*

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With two plates (figs. 6 and 7)

Summary

The viviparity recorded for this ascidian parallels that of placental mammals in reduction of the number and size of the eggs, the development of extra-embryonic membranes, and the prolongation of larval development with increase in the gestation period.

The ovum is alecithal, 25μ in diameter; it develops within an oviducal brood pouch in which the lining cells become distinctly modified and which remains attached to the parent for 5½ months.

Exposure of the embryo to the lumen of the pouch is brought about by rupture of the overlying follicle cells due to enzyme secretion by the dorsal-most cells of the blastula.

Endodermal development is precocious. At the gastrula stage the future oesophageal region, the future stomach, and a pair of endodermal tubes are distinguishable.

The paired endodermal tubes are unique in the ascidians. They are larval structures which disappear after metamorphosis and bear no relation to the functional stigmata of the oozooid. They resemble the branchial tubes of the Appendicularia, developing as blind outgrowths which later open through to the exterior; but in this species they open dorsally, not ventrally. They act as passive channels through which material from the pouch passes into the gut (a bolus being present from the gastrula stage onwards) and as anchoring strands for the ectotrophe.

This extra-embryonic membrane formed by elevation of the dorsal ectoderm ultimately practically envelops the embryo and becomes apposed to the lining of the pouch. It is extremely simple, exhibits no vascularization, makes no actual liaison with the oviducal epithelium, and shows little cell differentiation.

Extra-embryonic material reaches the embryo indirectly by diffusion across the ectotrophe (which unlike the remaining embryonic ectoderm secretes no test), and directly through the endodermal tubes. The latter method is the more important during early stages of development, the former during later stages.

The production of an extremely large and complex tadpole with numerous buds at an advanced stage of organogenesis testifies to the success of this type of viviparity.

Introduction

HYPSISTOZOA FASMERIANA (Michaelsen) is a compound ascidian belonging to the sub-family Holozoinae (Berrill, 1950). Michaelsen in 1924, studying material collected during a month when gonads were not discernible, placed this species in the genus *Distaplia* Della Valle. However, further investigations have shown that it does not belong to that genus and, in a survey of the Holozoinae (1953), the author erected the genus *Hypsistozoa* to house it. Four other members of the sub-family occur in New Zealand waters —*Distaplia taylori* Brewin (1950, 1951), *D. marplesi* Brewin (1952), and

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D. knoxi Brewin (1954), all of which are endemic, and *Sycozoa sigillinoides* Lesson, which has a circum-south-polar distribution.

As far as is known at present *Hypsistozoa fasmeriana* is endemic to New Zealand with a geographical range from Stewart Island in the south to Cape Kidnappers in the north (Michaelsen, 1924; Brewin, 1946, 1950, 1952*a*, 1953); it does not extend beyond the northern limits of sub-antarctic water (Deacon, 1937). It grows on coastal rocks about low-tide level in localities where water circulates freely and a good supply of both food and oxygen is available.

Much of the material on which the present paper is based was collected in the vicinity of the Portobello Marine Biological Station, Otago Harbour, where observations have been made over the last 10 years, aquarium and laboratory facilities having made possible a survey of the complete life history. The greater part of the investigation, however, was carried out in the Department of Zoology, University of Otago.

Appearance of Colony and Structure of Zooids

The colonies are capitate (fig. 1, A), the plane of division between head and stalk being slightly off the horizontal. The test is a clear light brown and the

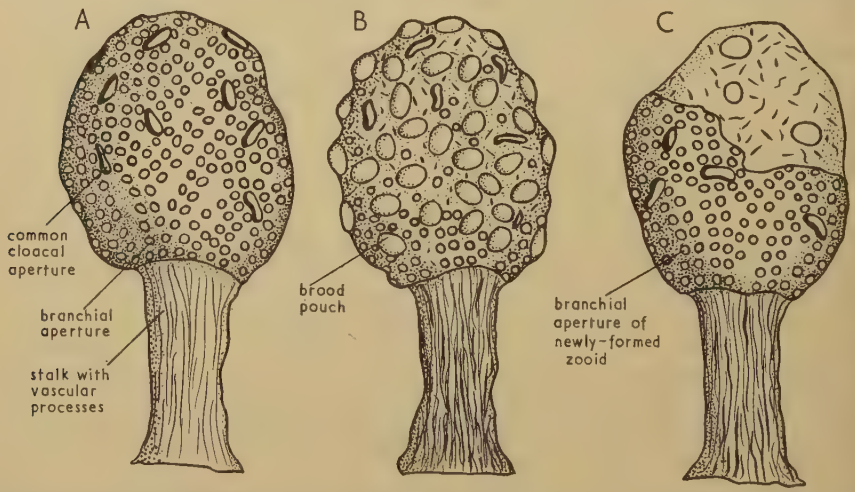

Fig. 1. A, normal appearance of the colony. B, colony in late October with brood pouches projecting and some of the distal zooids in a state of regression. C, colony in November, after tadpole liberation, showing newly formed zooids and test near the stalk, regressing zooids, and test at the distal end.

bodies of the purple zooids show through it by transparency. The zooids are arranged in branching systems which radiate around the common cloacal apertures, which are situated on the head region and mainly distally. The stalk of the colony contains the long posterior projections of the zooids, the vascular processes, and the buds derived from them.

Throughout the sub-family Holozoinae the zooid structure is fairly uni-

form. That of *H. fasmeriana* conforms to the general pattern as seen in the genera *Distaplia* and *Sycozoa* in form of the gut, position and mode of origin of the brood pouch, and structure and position of the cardiopericardium, but differs from it in shape and extent of the epicardium (a full description of which will be given in another paper) and in position of the gonads. In the Holozoinae the gonads are found typically in the abdominal region lying to the right side of the gut loop. A variation of this arrangement is seen in a small number of *Distaplia* species, the '*D. stylifera* group', in which the gonads are

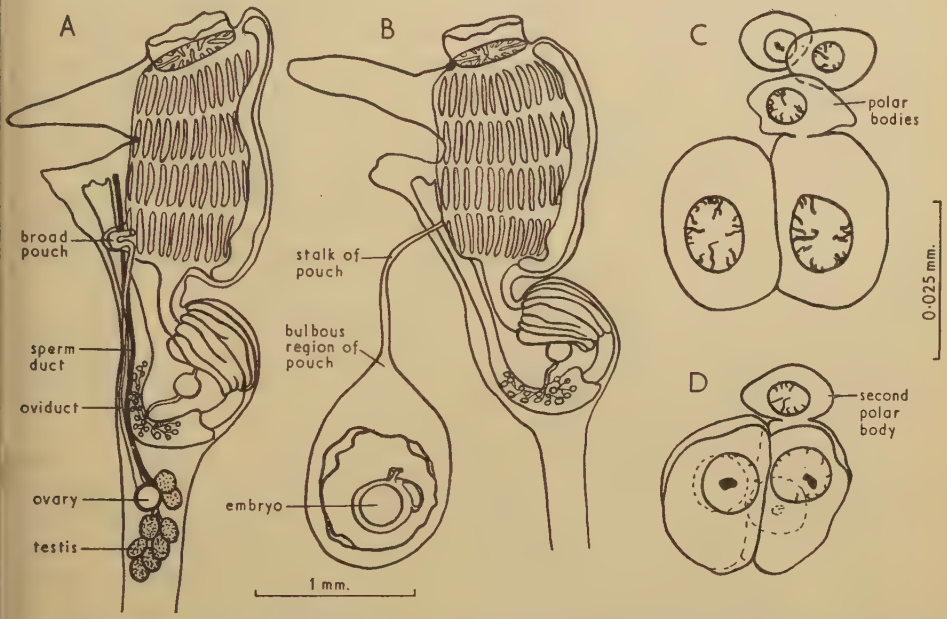

FIG. 2. A, zooid with gonads and brood pouch; egg not yet liberated from the ovary. B, zooid in which gonads have regressed, showing the attached brood pouch containing an embryo at the gastrula stage. C, two-celled embryo with polar bodies attached. D, four-celled embryo with second polar body attached.

contained in a sac-like diverticulum projecting from the abdominal region—a diverticulum which is quite independent of the vascular process. In *Hypsistozoa fasmeriana* a much more atypical arrangement is seen, the gonads lying not in the abdominal region but in the anterior end of the right side of the vascular process (fig. 2, A), and not in a sac-like diverticulum projecting into it as previously described (Brewin, 1946).

Each zooid is hermaphrodite. The testis consists of 8 to 16 pear-shaped lobes and from it the sperm-duct runs up the dorsal side of the zooid to open near the atrial siphon. The ovary produces only one egg. The oviduct runs parallel and dorsal to the sperm-duct (fig. 2, A) but is shorter than it and opens at the base of the atrial cavity. A week or more before the egg leaves the ovary, the oviduct grows in length with the result that a short hairpin bend develops near the distal end. This projects outwards and causes a sac-like outgrowth of

the zooid which appears on the right side close to the mid-dorsal line (fig. 2, A) and which acts as a brood pouch.

Annual Rhythm in the Colony

Reproductive organs are not discernible throughout the year. The testis which ripens in May continues to produce sperms throughout June and July and finally undergoes dedifferentiation. The single egg, which is very small, passes up the oviduct late in May, in June, or occasionally early in July, eggs maturing first in the large zooids near the distal end of the head of the colony and later in the somewhat smaller zooids near the proximal end. Both fertilization of the egg and embryological development take place in the brood pouch (fig. 2, B), which grows considerably and *retains its connexion with the parent zooid for approximately 5 months*—about $5\frac{1}{2}$ months being the time taken for the fertilized egg to develop into an active tadpole. Three weeks or less before the end of embryonic development, dedifferentiation of the parent zooid begins. The anterior ends of the zooids are affected first and, as they become reduced in size, the test overlying them sinks in. Thus a pitting of the surface of the colony occurs, leaving only the portions of test overlying the brood pouches in their original position, and the colony attains the appearance shown in fig. 1, B. As dedifferentiation proceeds mesenchyme cells migrate towards the posterior ends of the zooids and accumulate in the lower halves of the vascular processes. At this season the processes become more opaque and are very easily seen through the transparent test of the stalk of the colony. Late in October or early in November the tadpoles become active and by their tail movements rupture the walls of the brood pouches, break through the overlying test, and swim off. Within the colony three processes are occurring, dedifferentiation of the parent zooids, liberation of the tadpoles, and formation of new zooids. (A full description of the origin and development of colonial buds will be given in a further paper.) Each bud gives rise to one zooid, the branchial aperture of which opens at the junction of the head and the stalk of the colony, and the ectoderm of which secretes test. Zooids and newly formed test are thus added to the proximal end of the colony (fig. 1, C), whilst the old test, which is practically all that is left of the distal end, is resorbed; and by a combination of these phenomena a new head is established. This is accomplished often within 2 weeks of tadpole liberation. Considerable increase in the size of the colony takes place during the summer months, when the metabolic rate of the zooids is high and growth occurs rather rapidly. In the autumn the rate of budding decreases somewhat and towards the end of this season the zooids attain maximum average size, and reproductive organs become discernible. With maturation of the gonads the annual cycle of events in the colony, as shown in fig. 3, is recommenced.

A survey of the plankton of Otago Harbour was made during the years 1944–5 (Brewin, 1952*b*). In 1945 the peaks of phytoplankton appeared in April (autumnal peak) and July (spring peak). These were followed after intervals of approximately $2\frac{1}{2}$ months by peaks of zooplankton. Hence it is likely that in

most years a good supply of food for filter-feeding animals is available in this locality from April until the end of October, i.e. throughout the months during which gonad maturation and embryological development of *H. fasmeriana* occur.

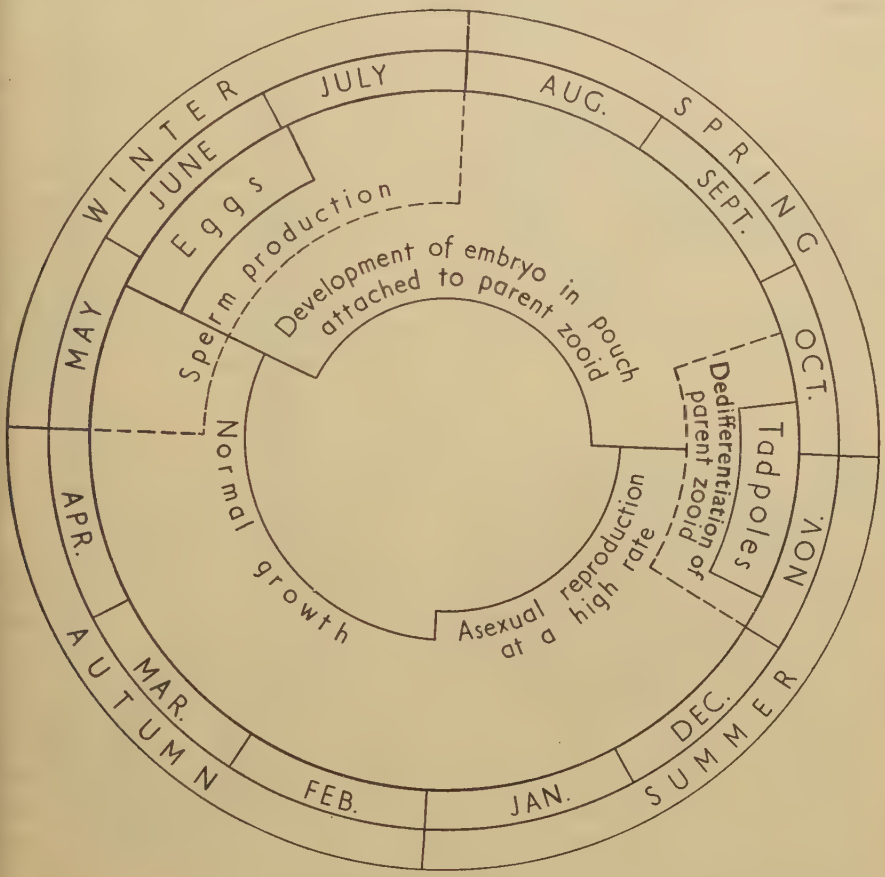

Fig. 3. Annual cycle of events in the colony.

The Brood Pouch

Bancroft's work in 1899 established clearly that in the Holozoinae, before the liberation of the first ovarian egg, there is a marked extension of the oviduct resulting in the formation of a projecting U-shaped bend.

In *Distaplia* and *Sycozoa* this becomes extensive with marked differentiation between the two arms (de Selys-Longchamps, 1913), the proximal arm remaining narrow while the distal one increases considerably in width, its cells being stretched to form a thin pavement epithelium. The proximal arm has merely an oviducal function, whereas the distal arm into which the eggs are driven by the action of a ciliated band of cells has the function of a brood

pouch. The embryos develop inside the egg membranes and their presence in the brood pouch does not bring about a change in the cell structure of the wall.

In *Hypsistozoa*, however, there is no differentiation between the two arms of the U-bend and their diameter is relatively uniform (fig. 4, A). Except for a band of ciliated cells near the proximal end, the wall is composed of simple

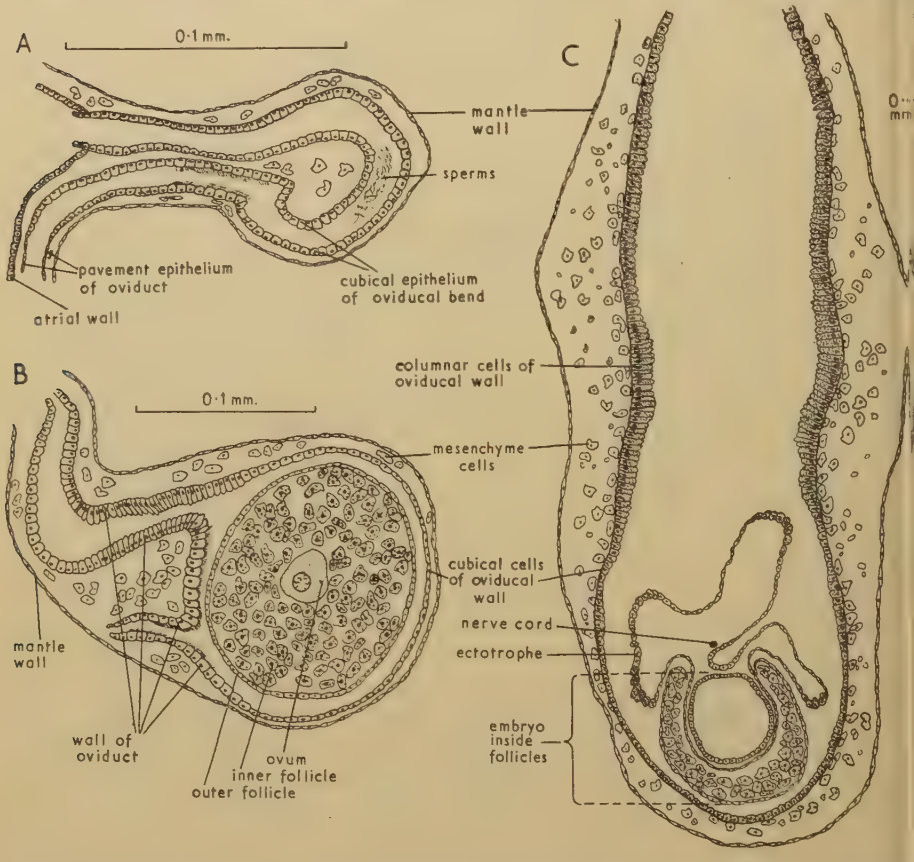

FIG. 4. The brood pouch. A, semi-diagrammatic view of newly formed pouch, showing oviducal bend lined with cubical epithelium. B, pouch after entry of the egg, showing extension of both mantle and peripheral part of the oviducal bend. Egg centrally placed among the numerous inner follicle cells. C, pouch at a later stage, showing extension of the blood sinus and modifications in the lining epithelium. The embryo is at the late gastrula stage.

cubical epithelium. Increase in the size of the bend with extension of the peripheral region is brought about by the entry of the egg and a brood pouch is formed, what was originally a U-shaped bend attaining the form of a single sac (fig. 4, B).

With expansion of the pouch, extension of the overlying mantle and of the blood sinus between the oviducal lining and the mantle wall takes place, and change is also induced in the structure of the wall of the pouch. Granules appear in the protoplasm of the cells and differentiation occurs, those cells

below and adjacent to the embryo becoming stretched and flattened, those above it becoming columnar (fig. 4, C).

That there must be some form of extra-embryonic nutrition in this species is apparent, since a large complex tadpole, measuring up to 6·8 mm in length and 1·6 mm in width in the head region and with 9 to 14 well-developed buds is derived from an egg only 25μ in diameter and devoid of yolk. The granular cytoplasm and the irregularities of the inner borders of the cells lining the occupied brood pouch suggest that these cells are selective and secretory in nature (figs. 6, F; 7, A). George (1939) showed that in the ascidians specialized blood-cells act as food-transporting agents. Examination of many sections of brood pouches cut at 1μ to 3μ failed to reveal evidence of amoeboid blood-cells passing from the sinus into the lumen of the pouch by squeezing through between the columnar cells of the pouch lining. There is evidence, however, that blood-cells tend to align themselves along the outside of the columnar cells (fig. 4, C) and it is most probable that the nutrient material carried by specialized blood-cells passes into the columnar cells by way of the blood-plasma.

Embryological Development

The ovum proper is alecithal and extremely small, measuring only 25μ in diameter. It is enclosed in two cell layers: (1) an outer follicle composed of a single layer of small flattened cells, and (2) an inner follicle composed of numerous irregularly shaped cells containing granules; these cells form a loose packing several layers deep between the ovum and the outer follicle (figs. 6, A; 4, B). There is no visible chorion.

Owing to the extremely small size of the egg and to the opacity of both the inner lining of the brood pouch and the inner follicle cells, orientation of the embryos presented great difficulty and all early embryological stages have been reconstructed from serial sections cut at 1μ to 3μ. The most satisfactory technique was found to be fixation in Bouin's fluid, impregnation in SSB3 wax (Watermann, 1939), and staining with Heidenhain's haematoxylin and eosin, or acid fuchsin methyl green (as recommended by Bancroft, 1899). Mallory's triple stain and chlorazol black were also useful for later developmental stages.

The first polar body is formed while the egg is still in the ovary, and may become separated from the ovum immediately or remain attached to it until after the formation of the second polar body. Upon maturation the egg leaves the ovary and passes up the narrow oviduct. During this passage the follicle cells are so compressed that the egg assumes an ovoid shape and only after entering the wider lumen of the pouch does it regain its spherical form.

As in other members of the sub-family, sperms brought into the zooid in the feeding current find their way to the brood pouch and fertilization may take place immediately after the entry of the egg. After penetration of the sperm the nuclear division responsible for the formation of the second polar body takes place and again cytoplasmic division may lag behind nuclear division, with the result that the second polar body may not separate off until several divisions of the embryo have occurred (fig. 2, C, D).

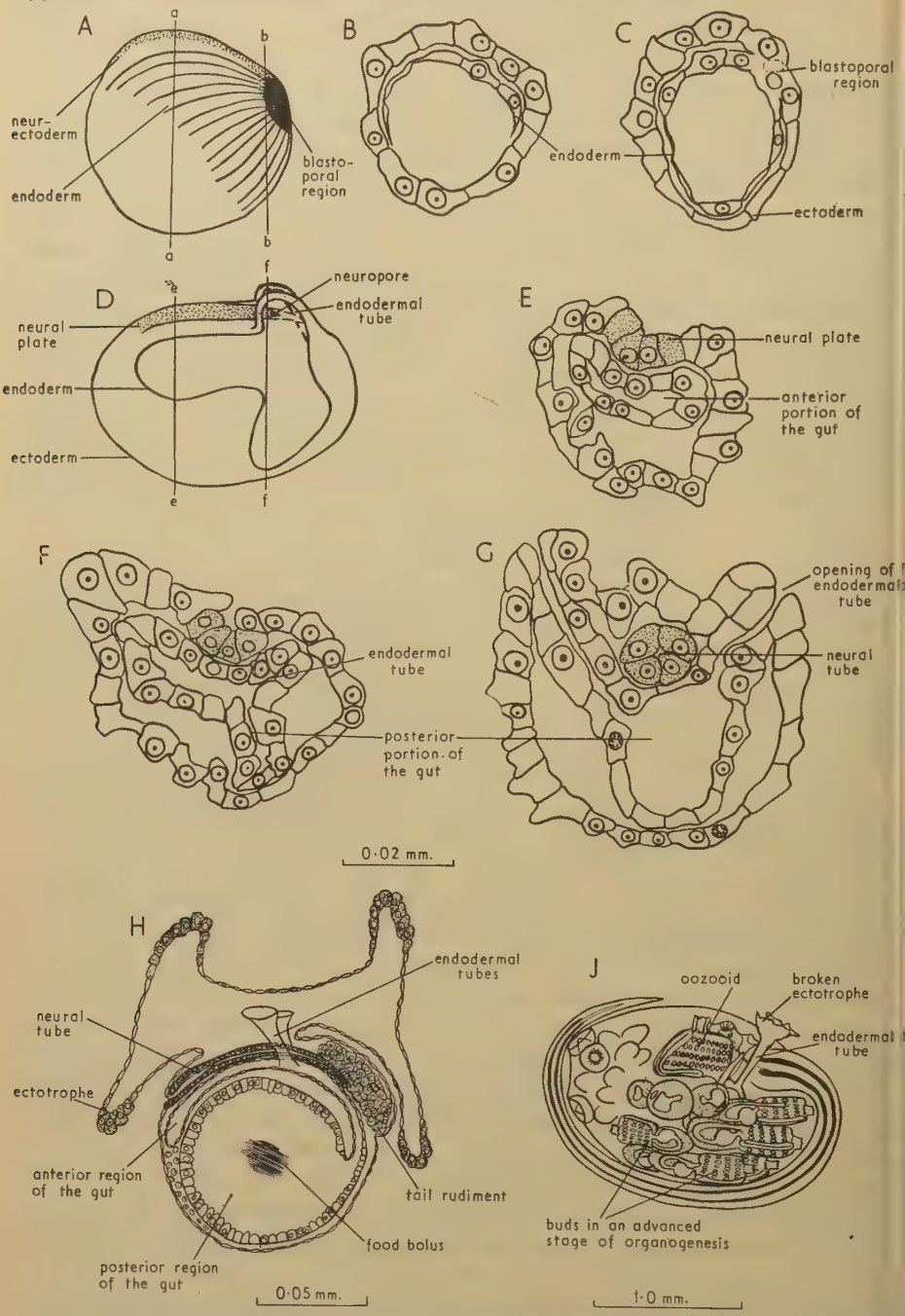

FIG. 5. A, diagrammatic reconstruction of a very early gastrula, showing the blastoporal region and the extent of the ectoderm. B, transverse section through A along the line *aa*; endoderm present in the dorsal hemisphere only. C, transverse section through A along the line *bb*; endoderm present in both hemispheres. Blastoporal region also shown. D, diagrammatic recon-

Cleavage is rapid. The first nuclear division is in the horizontal plane and the accompanying cytoplasmic division is meridional (fig. 2, C). The second nuclear division is also in the horizontal plane (fig. 2, D) and is followed by vertical divisions resulting in an embryo with 4 nuclei in the animal hemisphere and 4 in the vegetal. (The 8-nucleated stage is not achieved directly, 5-, 6-, and 7-nucleated stages being found.) Subsequent nuclear divisions are not easy to follow and cell outlines are not clearly distinguishable, but at the morula stage it would appear that there is little difference in size between the cells of the two hemispheres (fig. 6, A).

At the 30- to 36-celled stage the embryo is still spherical but has increased in diameter to about 32μ, a blastocoelic cavity 17μ or so in diameter having formed between the cells in such a way that the wall above is two or three cells thick, that below only one cell thick. With the appearance of the blastocoel the embryo abandons its central position among the follicles and moves dorsalwards with the result that only one, two, or at the most three inner follicle cells separate the dorsal surface of the blastula and the outer follicular layer (fig. 6, B).

Gastrulation begins after the sixth cleavage as in other ascidians but the order of development of the various presumptive areas is somewhat upset, growth of the endoderm being extremely precocious, that of the mesoderm and notochord tardy. Thus three distinct phases of gastrulation are recognizable—the early gastrula stage, the mid-gastrula, and the late gastrula.

An open blastopore formed by invagination characteristic of *Clavelina* (van Beneden and Julin, 1884) and of ascidians in general is unknown in the subfamily Holozoinae. In *Distaplia magnilarva*, on which extensive embryological studies were made by Davidoff in 1889, the egg is so heavily yolked that the archenteric cavity is occupied by large food-laden endoblastic cells and the blastopore is purely superficial. Gastrulation is by epiboly only. (Serial sections in the author's collection cut through embryos of *Sycozoa sigillinoides* show that the pattern of blastoporal formation and the mode of gastrulation are identical with those described by Davidoff for *Distaplia*.) In *Hypsistozoa* blastoporal reduction has been carried yet a stage further. No actual opening can be recognized and there is no true archenteron. There is, however, an active blastoporal region at the posterior end of the embryo (fig. 5, A).

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struction of the mid-gastrula, showing closure of the neural plate, development of dorsal extensions due to upthrusting of the endoderm, and differentiation of the anterior and posterior regions of the gut. E, transverse section through D along the line *ee*, showing the shallow anterior region of the gut. F, transverse section through D along the line *ff*, showing the deep posterior region of the gut and the dorsally directed closed endodermal tubes. G, transverse section through the posterior region of a mid-gastrula, showing the endodermal tubes opening to the exterior on each side of the neural tube and slight elevation of the dorsal ectoderm. H, the late gastrula in median optical section, showing the tail rudiment and the now spherical posterior portion of the gut with the narrow anterior portion encircling its dorsal surface. The endodermal tubes (superimposed) open into the anterior portion of the gut. J, a tadpole removed from a brood pouch, showing the advanced state of organogenesis reached by some of the buds and the presence in the oozooid of two different endodermal openings, the stigmata, and the endodermal tubes.

From the blastoporal region the endoderm arises precociously, growing at first more rapidly dorsally than ventrally (fig. 5, A, B, C). With further growth it completely encloses the greater part of the blastocoelic cavity, which is thus converted into the lumen of the embryonic gut.

As the egg carries no yolk as a food reserve, the energy necessary for the later stages of development of the embryo must be obtained from an extra-embryonic source and to make this possible the embryo is liberated by digestion of the follicle cells. Digestion of egg membranes by enzymes secreted by embryos at an advanced stage of development is not unknown among the ascidians (Berrill, 1929). In *H. fasmeriana* it commences at the early gastrula stage. The cells of the embryo that were the dorsal-most in the blastula increase in size, become vesicular, and finally rupture, liberating their contents on to the follicle cells nearby (fig. 6, D). That the ruptured cells contain digestive enzymes is apparent from the effects produced. The inner follicle cells in the vicinity show first swelling of the nucleus accompanied by facilitation of nuclear staining, secondly rupture of the nuclear membrane and shattering of the nuclear material (fig. 6, C, D), thirdly cytoplasmic swelling, and finally breaking of the cells into amorphous masses (figs. 6, E; 7, A). Rupture of the outer follicles may be effected directly by digestion or indirectly by the pressure exerted by the swelling of the inner follicle cells or by a combination of both methods, and as the result part, if not all, of the dorsal surface of the embryo becomes exposed to the fluid in the lumen of the pouch (fig. 6, E, F).

By the end of the early gastrula stage the embryo has become somewhat ellipsoidal, the longest diameter measuring approximately 38μ; the endodermal layer has encircled the greater portion of the blastocoelic cavity, and the dorsal surface of the embryo, part of which can be distinguished as the neurectoderm, lies exposed to the lumen of the pouch. It should be noted that the cells which form the true dorsal surface of the gastrula are the cells which at the blastula stage formed the roofing of the blastocoelic cavity, the cells dorsal to them at that stage, the enzyme secretors, separating off from the embryo at various stages of development.

The mid-gastrula stage is characterized by extension of the embryo and

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FIG. 6. Photomicrographs of embryonic stages of *Hypsistozoa fasmeriana*. Material fixed in Bouin, stained with Heidenhain's haematoxylin and eosin, and sectioned at 3μ. A, longitudinal section of a 24-celled morula. Polar bodies slightly above morula and to the left. Embryo removed from the outer follicle by about 6 inner follicle cells containing granules. B, median sagittal section through a blastula, showing the orientation of the blastocoel. Embryo removed from the outer follicular layer by only two or three inner follicle cells. C, illustration of the disintegration of the inner follicle cells dorsally to the embryo. The nuclei are shattered and have stained more deeply than those of the cells to the right of the embryo. D, median sagittal section through a late blastula, showing groups of inner follicle cells, dorsal to the embryo enclosed by the enzyme-secreting embryonic cells, which have ruptured and become goblet-shaped. E, longitudinal section of a mid-gastrula. Neurectoderm discernible on dorsal surface (left side as the embryo lies). Rupture of the follicles has occurred and the surface of the embryo is exposed to the fluid in the lumen of the pouch. F, portion of a longitudinal section of a brood pouch. On the left the columnar cells of the lining show granular cytoplasm and irregularities of the inner borders. Dorsally to the embryo can be seen enzyme-secreting cells and numerous follicle cells in process of digestion.

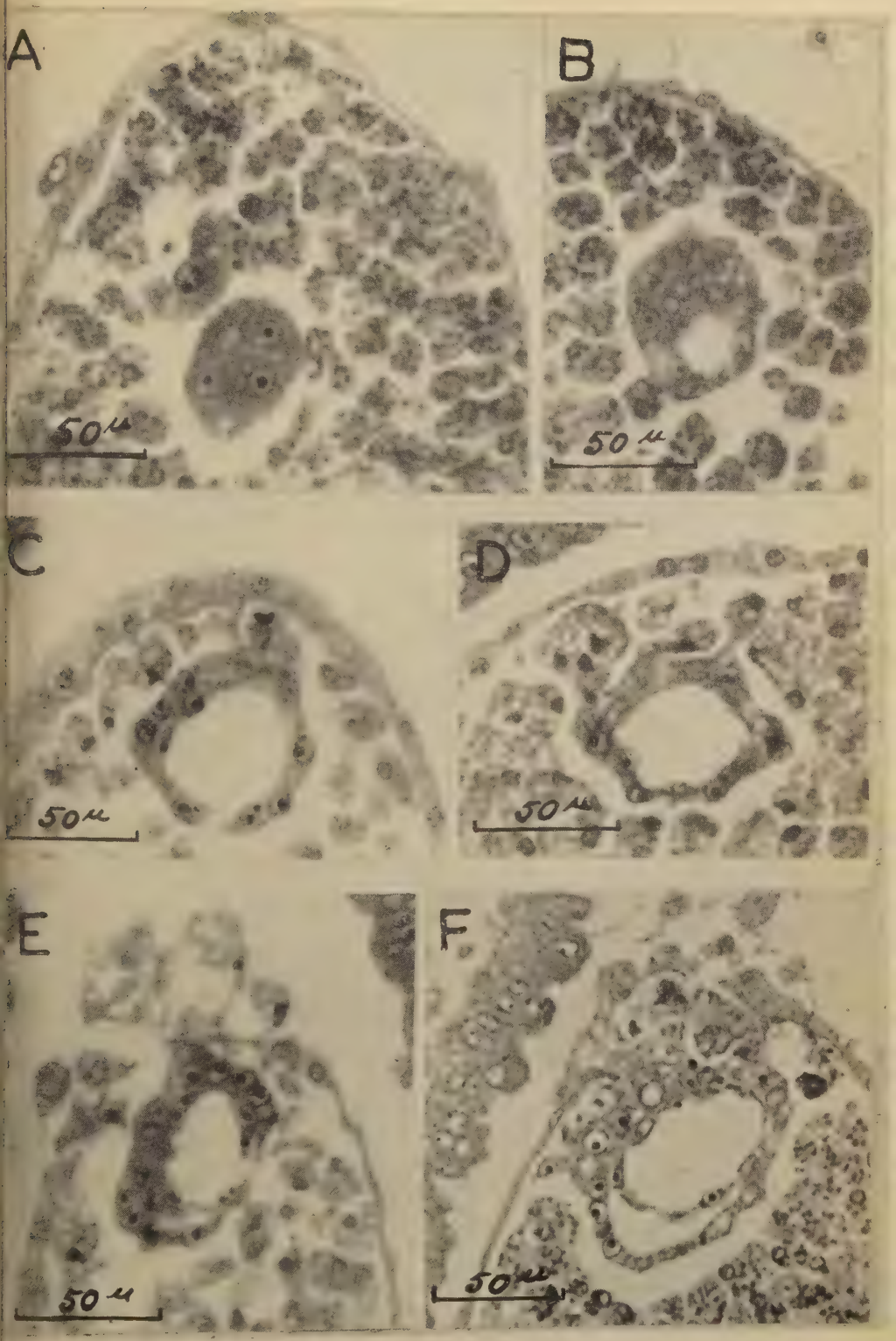

FIG. 6

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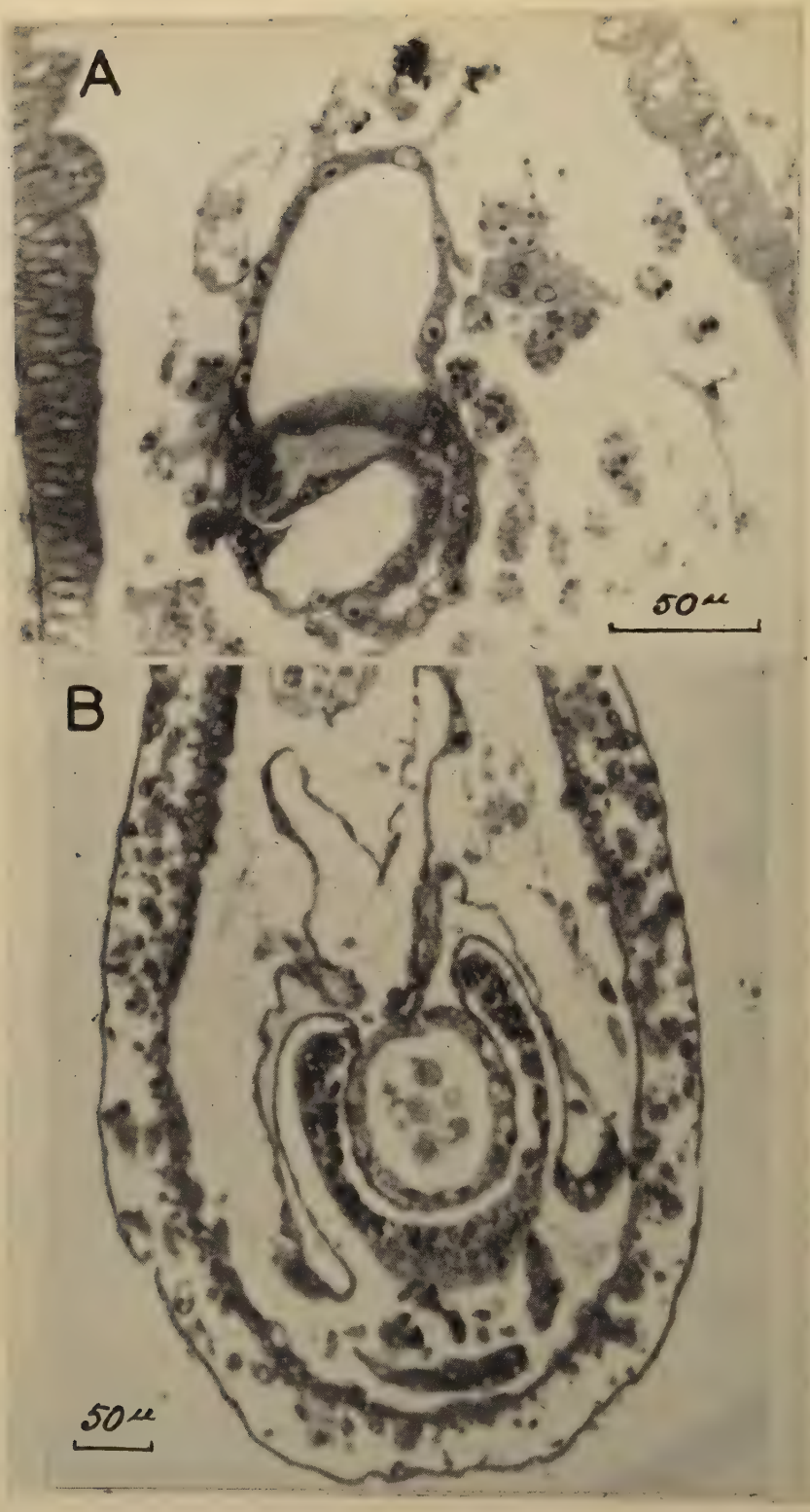

FIG. 7

B. I. BREWIN

elaboration of ectodermal and endodermal structures. In a manner identical with that described for *Clavelina*, the neurectodermal plate which was distinguishable on the dorsal surface of the early gastrula (figs. 5, A; 6, E) is rapidly converted into a neural tube with an anterior neuroporal opening. While closure of the neural tube is taking place, development of endoderm proceeds apace with the result that the depth of the endoderm at the posterior end of the late gastrula is far greater than that at the anterior end (fig. 5, D, E, F), and upthrusts of endoderm with consequent elevation of the overlying ectoderm develop on each side of the neural plate (fig. 5, D, F). These dorsally directed endodermal tubes later break through their ectodermal coverings and open to the exterior (fig. 5, G), so that the mid-gastrula has three external openings, the single median neuropore anteriorly and the two openings of the endodermal tubes posteriorly and dorso-laterally. With expansion of the embryo, fluid and cell fragments are drawn from the lumen of the pouch through the openings of the endodermal tubes and into the gut cavity so that a food bolus is present in the gut from the mid-gastrula stage onwards.

A secondary effect of the dorsal upthrust of the endodermal tubes is the elevation of the ectoderm adjacent to that immediately overlying them. This elevation is seen first in the region above the nerve cord (fig. 5, G). It is extremely vigorous and rapid and results in the whole dorsal ectoderm of the embryo growing up through the broken follicles into the lumen of the pouch (figs. 7, A; 4 C), to serve as an extra-embryonic membrane, henceforth referred to as the *ectotrophe*. The ectoderm of the ectotrophe is continuous with that of the embryo proper and is broken at two places posteriorly by the openings of the two dorsally directed endodermal tubes. The latter themselves show little growth in length and breadth and thus, in addition to functioning as intake channels for material from the lumen of the pouch, they serve also as attachment tubes anchoring the ectotrophe firmly to the embryonic gut.

Only after the neural tube, the endodermal tubes, and the ectotrophe are well established do the presumptive areas destined to form the mesoderm and notochord show sufficient development for a tail rudiment to be recognizable as such, and it is not until the late gastrula stage that this pushes out from the

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FIG. 7. Photomicrographs showing the ectotrophe and endodermal tubes of *Hypsistozoa fasmeriana*. Material fixed in Bouin, stained with Heidenhain's haematoxylin and eosin, and sectioned at 3 μ. A, median sagittal section of an embryo, showing elevation of the dorsal ectoderm as an ectotrophe, on the left of which can be seen inner follicle cells in all stages of digestion from nuclear swelling only to complete shattering with liberation of the granular contents. The gut of the embryo is ↄ-shaped and surmounted by a portion of the neural tube. Cells lining the brood pouch are shown at the edges of the plate. B, longitudinal section through the ventral portion of a brood pouch, showing the position of the late gastrula. The ectotrophe with its two lateral expansions passes up towards the neck of the pouch and down towards the base, enclosing the embryo, most of which is still surrounded by follicle cells. It makes no actual contact with the lining of the pouch and only the cells at the peripheries of the extensions show differentiation. The two endodermal tubes are cut so that their external openings are visible, but the internal opening to the oesophagus is shown only in the tube on the right. Material from the lumen of the pouch is present in the tubes and also in the large stomach, which occupies the greater part of the embryo proper. The small neural tube lies immediately dorsal to the oesophagus.

blastoporal region as a short, rounded bulge, disrupting the otherwise spherical shape of the embryo proper which has by now extended to approximately 100μ in diameter (fig. 5, H). Mesodermal development is not confined to the tail region. Wandering cells from the blastoporal region migrate down to fill the space between ectoderm and endoderm and by the end of gastrulation all three cell layers are clearly recognizable both in the trunk region and in the tail.

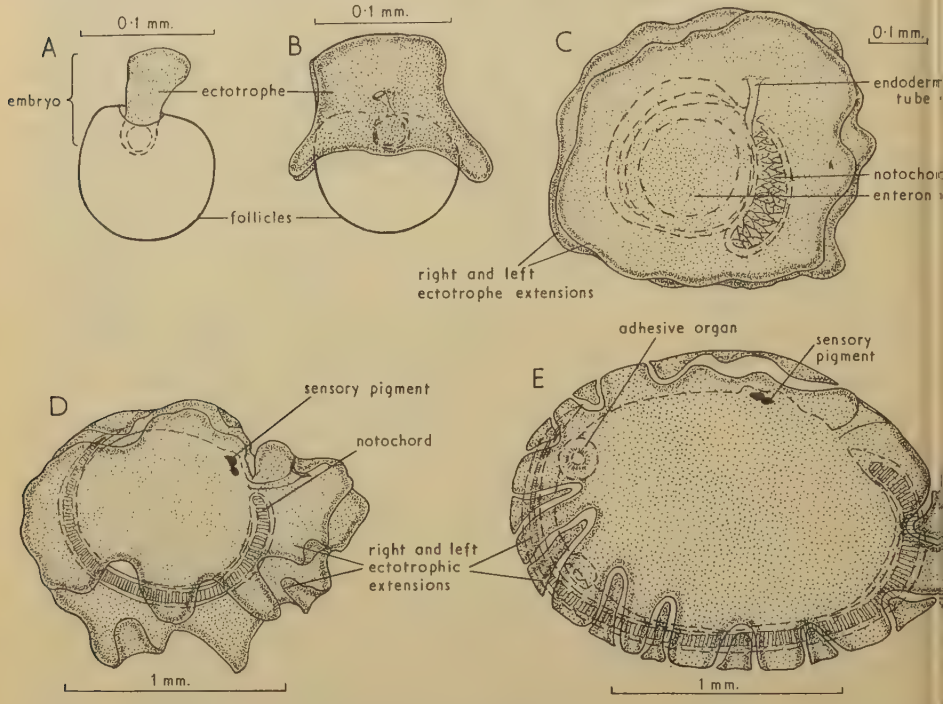

FIG. 8. Embryos removed from the pouch (ectotrophe stippled). A, the early gastrula enclosed in follicles, the finger-like ectotrophe alone projecting. B, a slightly older gastrula with the two lateral ectotrophic extensions partly enclosing the follicles. C, a late gastrula, with tail rudiment, completely enclosed between the circular ectotrophic extensions which are far more extensive than the embryo. D, an early tadpole. The ectotrophic extensions are still more extensive than the embryo and growth at the peripheries has resulted in outgrowths so arranged that those of the right extension fit recessions in the left. E, a tadpole with the tail fully extended. The area of the tadpole closely approximates to that of the ectotrophe, the two extensions of which barely meet around the embryo.

The endodermal arrangement of the late gastrula is unique. The shallow anterior portion has developed into an extensive, narrow, tube-like structure (the future oesophagus) which encircles the dorsal half of the posterior portion (the future stomach) which has become a large spherical sac containing food and occupying the greater part of the embryo proper. It is apparent that the two dorsally directed endodermal tubes open into the posterior end of the future oesophagus and not into the future stomach.

At the late gastrula stage the ectotrophe loses its simple tube-like form (fig. 8, A) and extends up towards the neck of the pouch and down around the

follicles which still surround the embryo proper (fig. 8, B). Elevation being somewhat restricted in the median dorsal direction by the presence of the two endodermal tubes, expansion is mainly lateral and results in disk-like extensions pushing down, one on each side of the embryo, to occupy the space between it and the wall of the brood pouch (fig. 7, B). Cell differentiation also occurs in the ectotrophe, the cells at the peripheries of the lateral extensions being clearly distinguishable by their somewhat columnar form, large size, granular appearance, and deeply staining propensities. Like the columnar granular cells lining the brood pouch, these cells take on a bright blue coloration with Mallory's triple stain after Bouin fixation and are perhaps food-absorbing cells. During gastrulation increase in the area of the ectotrophe far exceeds increase in the area of the embryo proper and at the end of this period the latter appears as a small spherical structure centrally situated between the two large lateral extensions of the ectotrophe, which have become almost circular in outline (fig. 8, C).

The embryological development of *H. fasmeriana* like that of most viviparous species is extremely rapid and in this species gastrulation is completed usually about one week after fertilization of the egg.

Larval Development

After the extremely short embryological developmental period there is a greatly prolonged period of larval development which occupies approximately 5 months and results in the production of a very large and extremely complex tadpole. A detailed description of larval development will appear in a later paper but accounts of stolonial budding, ectotrophal development, and size attainment are included here.

A stolon containing an epicardium and destined to give rise to buds is known to develop on the left side of the post-larva in the sub-family Holozoinae. It has been recorded for several species of *Distaplia* (Berrill, 1935; Brien, 1939; and others) and for *Sycozoa tenuicaulis* (Brewin, 1953); but whereas in those genera it is short and narrow and contains comparatively few mesenchyme cells, in *Hypsistozoa fasmeriana* it is long and wide (width, *H. fasmeriana* 235μ; *Distaplia magnilarva*, 45μ) and densely packed with opaque, granular mesenchyme cells. In *Distaplia* and *Sycozoa* strobilation results in the formation of a few small probuds and residual buds, none of which develops directly into a blastozooid. The residual buds usually degenerate and the probuds undergo further division with formation of two or three very small definitive buds which in the larval stage manage to attain only a low degree of organogenesis. In *Distaplia rosea* it has been shown that even the largest of them cannot become a functional blastozooid until energy (necessary for the completion of its organogenesis) is made available by dedifferentiation of the oozooid of the young colony (Berrill, 1948). In *Hypsistozoa* strobilation results in successive formation of 9 to 14 large buds, all of which develop directly into blastozooids and the majority of which attain a high degree of organogenesis in the larval stage (fig. 5, J). They complete their development

during metamorphosis and become functional as blastozooids *together with* the oozooid of the young colony.

Early in the larval period invagination begins at the junction of the ectotrophic and true embryonic ectoderm and gives ectodermal coverage to the greater part of the dorsal surface of the embryo, only a small area in close proximity to the endodermal tubes remaining free from close ectodermal investment. The same invagination is responsible for the almost complete separation of the extra-embryonic membrane and the embryo proper and when test secretion occurs (at approximately the same time as outgrowth of the larval stolon) it is very noticeable that the ectotrophic ectoderm is not involved. It is specialized ectoderm and alone remains uncovered by test. In the early larval period the area of the embryo proper is still slightly less than that of the lateral ectotrophic extensions in which the growth-rate becomes unequal in the various sectors, with the result that the periphery of each extension is produced into blunt outgrowths so arranged that those of the opposing extensions interdigitate (fig. 8, D). In the later larval period the area of the embryo proper becomes as great as, if not greater than, that of the ectotrophic extensions and it is just barely possible for the interdigitating peripheries of opposing extensions to meet around the large fully developed tadpole (fig. 8, E). The lumen of the brood pouch is fully occupied during the later stages of larval development and the ectotrophic membrane lies hard up against the columnar lining of the pouch. During liberation of the tadpole (brought about by muscular movements of the larval tail) the ectotrophe is usually broken off close to the endodermal tubes and may either remain in the ruptured brood pouch for a time or immediately drift away on the tide. A few remnants of the ectotrophe, however, usually adhere to the endodermal tubes of the free-swimming tadpole and together with them project from the dorsal surface like an umbilical remnant.

The tadpole of *H. fasmeriana* as well as being extremely complex is without doubt the largest recorded for the sub-family Holozoinae, being almost twice the size of that of *Distaplia magnilarva* and three times the size of that of *Sycozoa sigillinoides*. The average measurements attained (based on the study of 50 tadpoles) are: head region, 2·9 mm long, 1·6 mm wide; tail region, 3·9 mm long, 1·0 mm wide.

The comparatively large size and the great complexity attained by the larva of *Hypsistozoa fasmeriana* bear strong witness to the success of the particular type of viviparity characteristic of this ascidian.

Egg-size and Egg-number in the Holozoinae

From examination of egg-size and egg-number in at least 20 individuals of all species of the sub-family Holozoinae recorded from Australia, New Zealand, and Japan the following points emerge:

1. In the ovoviviparous genera *Distaplia* and *Sycozoa* the eggs are heavily yolked and comparatively large, 280μ to 520μ in diameter. In the viviparous genus *Hypsistozoa*, they are devoid of yolk and extremely small.

2. In *Distaplia* and *Sycozoa* egg-size increases at the expense of egg-number. In each genus the species with the smallest egg has the largest average number of embryos per brood pouch (*Distaplia taylori*, egg-diameter, 290μ, average number of embryos 3; *Sycozoa sigillinoides*, egg-diameter 280μ, average number of embryos 6). Moreover in each genus there are species with eggs of large diameter (*Distaplia stylifera*, 520μ; *D. australensis*, 480μ; *Sycozoa cerebriformis*, 370μ) in which the number of embryos in the brood pouch does not exceed one, though more than one egg is present in the ovary. In these species once the first ovum is liberated from the ovary, development of the remaining ova is suppressed (Brewin, 1953). It is unlikely that suppression of this type

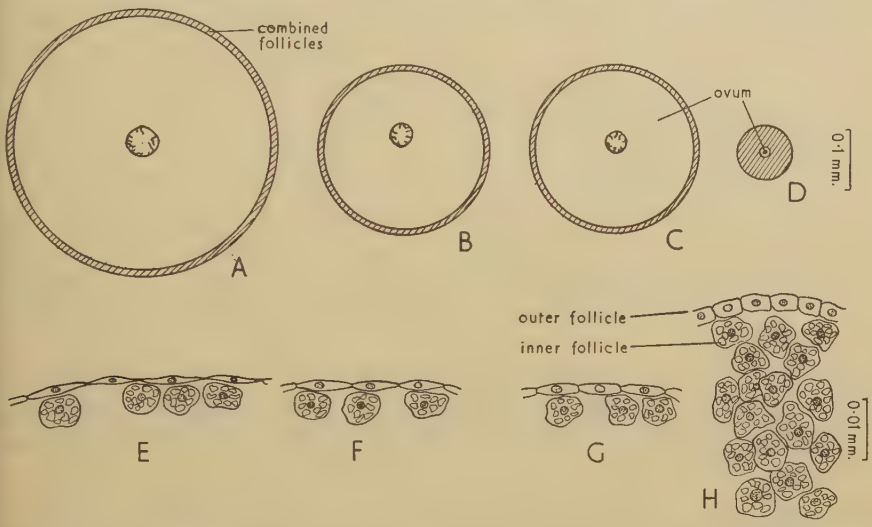

FIG. 9. A–D, eggs of *Distaplia marplesi*, *D. taylori*, *Sycozoa sigillinoides*, and *Hypsistozoa fasmeriana* respectively, to show comparative size of the eggs and depths of the follicular layers (cross-hatched). E–H, outer and inner follicles of *Distaplia marplesi*, *D. taylori*, *Sycozoa sigillinoides*, and *Hypsistozoa fasmeriana*, showing comparison between the shapes of the outer follicle cells and the arrangement of the inner follicle cells in the four species.

would occur unless the chance of survival of the single embryo were very great and it is concluded that such suppression is indicative of long-established and successful ovoviviparity. In the genus *Hypsistozoa* suppression of ova has been carried a stage further so that not only is one ovum only liberated but one ovum only is produced in the ovary.

3. In all species of *Distaplia* and *Sycozoa* the follicular layers surrounding the ovum are alike as regards total depth and arrangement of cells (fig. 9, A, E; 9, B, F; 9, C, G), the outer follicle consisting of stretched and flattened cells and the inner consisting of a single layer of irregularly shaped cells with granular inclusions. In *Hypsistozoa* (fig. 9, D, H) the combined depth of follicle, 38μ, is about 6 times that seen in *Distaplia* and *Sycozoa* species, the increase in depth being due mainly to the fact that the inner follicle cells form not a single layer but a loosely packed aggregation and only in very small measure to the more

cubical nature of the outer follicle cells. Berrill (1950) points out that cell numbers in the tadpole are rigid. If cell numbers in the follicular layers are equally rigid, reduction of egg-size should result in (*a*) absence of tension on the outer follicle cells, so that they are not stretched and flattened but remain cubical, and (*b*) rearrangement of the inner follicle cells to fill the space between the outer follicular layer and the very small ovum. The arrangement of the follicles of *Hypsistozoa fasmeriana* fits in exactly with this conception and support is thus given to the view that reduction of yolk in the egg is secondary.

No member of the Holozoinae has yet been found in which the developmental history forms a link between that of the heavily yolked species and that of *H. fasmeriana*. It is concluded that the sub-family Holozoinae has had a long evolutionary history of highly successful ovoviviparous development leading to reduction in egg-number and that the highly successful viviparity shown by *H. fasmeriana* probably represents the culmination of an evolutionary trend, of which the exact origin remains obscure but which can be traced back to a heavily yolked ancestral form showing reduction in egg-number.

Weekes (1935), studying viviparity in the reptiles, reached the conclusions that viviparity is not brought about primarily by lack of yolk content in the egg and that climatic influences may be a contributory or even the main cause. Harvey (1928) pointed out that there is a convergent association between the low temperatures of the environment and the frequency with which large-yolked eggs and the accompanying ovoviviparity or true viviparity appear in marine animals and it is possible that the coldness of its environment has been a contributory factor in the establishment of viviparity in *H. fasmeriana*.

The Ectotrophe

The viviparity of *H. fasmeriana* does not owe its success to the formation of a definite placenta. No actual fusion or even interdigitation takes place between embryonic and parental tissue, the ectotrophic ectoderm lying merely in apposition to the modified oviducal wall, the closest association being towards the end of the larval period when the lumen of the pouch can barely accommodate the huge tadpole and its ectotrophal wrapping.

From the anatomical point of view the extra-embryonic membrane of *H. fasmeriana* is extremely simple and fulfils the conditions that Hill (1922) suggested would be found in the first beginnings of viviparity—absorption by the embryo taking place across an ectodermal layer which exhibits no vascularization and which makes no actual liaison with the uterine epithelium. In some amniotes at a very early stage of embryonic development absorption takes place across an extra-embryonic chorion which makes no actual contact with the oviducal epithelium (Pearson, 1948). Though the chorion of the amniotes may resemble the ectotrophe of *H. fasmeriana* in that in both the extra-embryonic membrane concerned is ectodermal in origin and in that in both it acts as a simple membrane across which absorption of the uterine fluid takes place, the presence of a well-organized blood system in the somatic mesodermal portion of the chorion permits of rapid transport of material to and

from the embryo and renders the efficiency of viviparity greater than that of *H. fasmeriana*. In the latter, with no well-defined blood system and no concentration of mesenchyme cells beneath the ectotrophe, exchange of material between parent and offspring must be extremely slow. Also throughout development the ectotrophe remains relatively simple, with a few cells at the peripheries of the extensions undergoing differentiation but none showing syncytial or trophoblastic activities.

As an extra-embryonic membrane the ectotrophe is structurally unique, there being nothing like it in the development of chordates, protochordates, or the viviparous arthropods. It can best be compared with the dorsal region of the trophoblast of amniotes before the somatic mesoderm becomes applied and a chorion is formed.

In accordance with the position it occupies the ectotrophe is ideally situated as a medium for gaseous exchange and no doubt respiration is one of its most important functions. Though this membrane appears very early in development it is possible that it is less important at the early embryonic stages than it is after test secretion by the larva, when the ectoderm of the ectotrophe alone remains uncovered. The outer surface of the test must by its very nature be semi-permeable only, and at this stage the importance of the thin-walled ectotrophe as a diffusion region for the larva must increase.

The Endodermal Tubes

The endodermal tubes of *H. fasmeriana* are unique in the ascidians. Endodermal tube-like outgrowths develop in a similar manner in *Oikopleura dioica* (Class Appendicularia) (Delsman, 1910–12) but differ in arising from the pharyngeal region (not the oesophageal), in breaking through the ectoderm ventro-laterally (not dorso-laterally), and in becoming functional, ciliated, branchial tubes.

The egg of *Hypsistozoa fasmeriana* being without yolk, it is essential that nutritive material from an outside source be made available to the embryo at a very early stage. As the endodermal tubes function almost immediately after breaking through to the exterior as passive agents for the passage of material from the lumen of the pouch into the gut of the embryo, nutritive material can reach the embryo not only at a very early stage (the mid-gastrula) but also by an extremely direct method. Intake by this method is dependent upon the rate of increase of the gut cavity which is greatest during the early embryonic stages, when the area of the ectotrophe is still small, and it would appear that in the development of *Hypsistozoa* the two methods of absorption of nutrient material are complementary. Early in the gestation period the more important is direct intake through the endodermal tubes, whereas later it is the slower method of diffusion through the extra-embryonic membrane, the area of which increases greatly during larval development. The endodermal tubes have yet another function: they serve as anchoring strands connecting the ectotrophe to the larval endoderm.

It is interesting to find that development of endodermal openings to the

exterior occurs during embryonic development in two different classes of the Urochordata—the Ascidiacea and the Appendicularia—and that the resulting endodermal tubes perform widely different functions. In *Oikopleura* they serve as channels through which water is driven out of the gut. In *Hypsistozoa fasmeriana* they serve as passive channels by which food is brought into the gut and also as anchoring strands for the ectotrophe. In the latter species they disappear entirely after metamorphosis and bear no relation to the functional stigmata of the oozooid, i.e. they are essentially larval structures. The last statement holds also in the Appendicularia, all members of which are considered specialized neotenous larval types. It would thus appear that endodermal tubes have arisen independently at least twice in larval stages in the Urochordata, and probably, considering the Stomochorda, several times before the chordates settled down to the development of the typical gill-slit arrangement.

The Viviparity of *H. fasmeriana*

In the majority of comprehensive works on the Ascidiacea many viviparous species are listed, but the majority are ovoviviparous. True viviparity with the development of mechanisms for transference of material from parent to offspring and conversely is rare.

Two types have been recorded previously, and in both the embryos develop in the atrium. In the first type the atrial wall becomes thickened to form a cup-like maternal portion of a placenta, an aggregation of follicle cells forming the embryonic portion. This arrangement was described very briefly for three of the Synoicidae by Salensky (1892) and no details of development are available. In the second type, recorded for *Botrylloides leachi* by Ärnbäck-Christie-Linde (1921, 1923), a pouch-like invagination arises in the wall of the atrium. Berrill (1947) has shown that the cells lining the invagination become columnar, granular, and vacuolated, and from their plasmodial ends secrete a coaguable substance into the interior of the pouch; but no modifications of embryonic tissues in connexion with viviparity have been recorded, and Berrill in 1935 reported that the course and time of development of the embryo of *B. leachi* are practically the same as those of the embryo of *Botryllus schlosseri*, in which the egg is of larger diameter and no histological modification of the atrial wall takes place. (It is usual in successful viviparity for cleavage and gastrulation to be passed through with great rapidity so that the earliest use may be made of the extra-embryonic nourishment available.)

In *Hypsistozoa fasmeriana* the embryos develop in a specialized pouch of the oviduct, the lining cells of which become differentiated, with the appearance of deeply staining granules in their cytoplasm and the development of irregularities on their inner borders which suggest that they have become selective and secretory in nature. The egg is devoid of yolk and the embryonic tissues are greatly modified with (1) formation of endodermal tubes which open through the ectoderm and allow passage of nutrient material into the embryo, and (2) the formation of an ectodermal extra-embryonic membrane

which becomes apposed to the oviducal lining of the pouch. The early embryological stages are passed through very rapidly and are characterized by precocious development of endoderm and precocious secretion of digestive enzymes. The latter are responsible for clearing away of the follicles and exposure of the embryo to the fluid in the lumen of the pouch. In this species extra-embryonic nourishment is available from an early stage and larval development, which is greatly prolonged, results in the production of a very large and extremely complex tadpole.

There can be no doubt that in the Ascidiacea the highest form of viviparity is shown by *H. fasmeriana* and it would appear that in this class as in the other chordates the most successful viviparity is intra-oviducal.

Viviparity of a very different type from that of *H. fasmeriana* is seen among the Urochordata in the Salpida. Here the egg-membrane ruptures at an early stage and the follicle cells form a placental knob which becomes bathed in blood from the oviducal tissues. There is no formation of extra-embryonic membranes. (The enveloping membranes of the thecogone salps, the amnion and the chorion, are of atrial, not embryonic origin.)

Though no doubt the similarities between the viviparity of the placental mammals and that of *H. fasmeriana* are due mainly to functional adaptation, it is extremely interesting to find in a lowly member of the chordate line a type of viviparity which parallels very closely that of the highest members of the same line. Development in both cases is characterized by (1) reduction in egg-size and egg-number, (2) absence of an open blastopore and a true archenteron, though no yolk is present in the egg, (3) precocious development of endoderm (in *H. fasmeriana* this arises at a very early stage in gastrulation, but not before it, by delamination, as in mammals), (4) growth of extra-embryonic membranes, (5) acceleration of cleavage and gastrulation, and (6) prolonged larval development with increase of the gestation period.

I wish to express my gratitude to the University of New Zealand Research Grant Committee for a grant for photomicrographic equipment; to the staff of the Portobello Marine Station for the collection of material at regular intervals; to Dr. W. Van Name, Dr. T. Tokioka, the Discovery Committee, the Australian Museum, and Mrs. L. Marsh of West Australia for material of *Distaplia* and *Sycozoa* species; and to the late Sir W. B. Benham, Dr. P. Brien, and Dr. Ärnbäck-Christie-Linde for gifts of relevant literature.

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On the Origin of the Electric Organ in *Malapterurus electricus*

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With three plates (figs. 2, 3, and 4)

Summary

The present study was made on two small specimens of *Malapterurus electricus*, of standard lengths 11·4 and 12·7 mm. As is well known, the postembryonic growth of electric organs in *Malapterurus* and other electric fishes takes place by an enlargement of the electric units and not by an increase in the number of the electric plates. In the present material, however, there is a multiplication of electric tissue elements in the rostral portion of the electric organ. The structure of this multiplication zone is described. In the anterior region the connective tissue membranes which surround the two halves of the electric organ form structures similar to tendons which are fixed to the ventral surface of the shoulder girdle on each side of the median line. A small deficiency on each side in the muscular wall in the same region was observed in adult specimens by Maurer. This deficiency is more evident in the young specimens studied in the present paper and it is covered from the outside by the multiplication zone of the electric organ. In this place the electric nerve joins the electric organ. The giant electric cell, the surface of which is penetrated by intracellular capillaries, is situated in the anterior part of the spinal cord and its axon emerges with the third ventral spinal root. In the multiplication zone the connective tissue membranes are completely independent of the dermal connective tissue and the space between the electric organ and the skin is of a conventional subdermal type. These circumstances strongly indicate that the electric organ is of myoblastic origin in *Malapterurus* as in all other electric fishes known in this respect. Nothing has been observed which supports the idea of an adenoid origin of the electric organ in *Malapterurus*.

Contents

Introduction

In respect of all electric fishes except *Malapterurus electricus* facts are known which more or less definitely indicate that the electric organs have evolved phylogenetically from what were originally striated muscles (Babuchin, 1870, 1872; Ewart, 1889, 1892; Maurer, 1904; Böker, 1937; Rauther, 1937; Ballowitz and Schmidt, 1938; Nachmansohn, 1955; Schnakenbeck, 1955). In the case of *Malapterurus* other sources have been assumed. Fritsch (1883, 1887; see also Bridge, 1904; Schnakenbeck, 1955) believed that the electric organ in this fish arises from glandular elements of the epidermis, mainly, as it appears, because of the superficial position of the electric organ and the

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intimate connexion between the electric organ and the skin in these fishes. Rauther (1937) accepted the idea but pointed out the difficulty of explaining how intra-epithelial cells could change into a subcutaneous position. He noticed, however, that such a position was achieved by the cells of the axillary gland, which is found in many silurids. Such a gland is missing in *Malapterurus*, and Rauther advanced the hypothesis that the electric organ had evolved from this gland. He stressed the fact, however, that he had failed to produce evidence which would support this assumption, that is to say, a connexion between the electric organ and the epidermis in the axial region or an indication that such a connexion had existed earlier during the development. Stuart and Kamp (1934) mention the possibility that the electric organ of *Malapterurus* may have evolved from smooth musculature.

Ballowitz (1899) laid stress upon the fact that there are great similarities in histological structure between the electroplax of *Malapterurus* and that of other electric fishes. Consequently he considered that a muscular origin ought to be assumed also in the case of the electric organ of *Malapterurus*. Later, Ballowitz considered the origin to be doubtful (1938). Maurer (1913) observed that there seems to be a small deficiency in the anterior ventral portion of the musculus obliquus internus and suggested—with great reserve, possibly due to the fact that Ballowitz's original idea had not been generally adopted—that the electric organ might be the result of a change in this missing muscular portion. However, the key to the problem is missing, as the embryonic development of the electric organ of Malapterurus is unknown. The smallest specimens hitherto examined are 5–6 cm in length (Babuchin, 1877).

The assumption of an adenoid origin of the electric organ in *Malapterurus* is to some extent supported by the fact that the mode of function of this organ differs from that of other electric fishes (see Garten, 1911; Rauther, 1937). Physiologists and biochemists, on the other hand, explain the biochemically aberrant process behind the electric discharge of *Malapterurus* as due to its different source of origin (Augustinsson, 1955; Nachmansohn, 1955). Obviously, however, the discussion should be carried on only in one of these two directions.

Material and Method

The present investigation was performed on two small specimens of *M. electricus* (Gmelin) from the Gambia River, of standard lengths 11·4 and 12·7 mm in the fixed condition (see Johnels, 1954). The specimens were fixed in Bouin's fluid. They were sectioned transversely at 6 μ and 10 μ respectively. The stain was Heidenhain's Azan (Romeis, 1948). In the case of the smaller specimen there was partial combination with silver impregnation according to Bodian (1936, 1937).

Results

On the exterior surface the electric organ of *Malapterurus* is covered with a fibrous connective tissue membrane which is intimately connected with the dermal layer of the skin. (For a complete description of the electric organ see

Ballowitz, 1899, 1938; Garten, 1910; Rauther, 1937). Towards the interior there is also a layer of fibrous connective tissue. Between this interior membrane and the underlying muscular system there is intercalated a layer of loose connective tissue with very delicate reticular elements. Because of this the skin and electric organ may be easily stripped off the body as one unit, and this—together with the fact that after this process the rest of the body is very similar to what is left when only the skin is taken from the body of other kinds of fish—seems to have originally induced the idea that the electric organ really belonged to the integumental system (see Fritsch, 1883). In the two small specimens examined in the present investigation there are ruptures in this delicate tissue between the muscular system and the electric organ (fig. 3, B). These ruptures were evidently formed already during the fixing process. It seems probable that the function, at least in part, of this layer of loose connective tissue is to permit a certain freedom of movement between the muscular system and the thick cover formed by the electric organ and the skin.

In adult specimens the electric organ is said to cover the body from very close to the posterior part of the head to a level slightly posterior to the rostral parts of the dorsal (fat) and anal fins. Bilharz (1857) observed that, in front of and particularly behind the electric organ, the space between the skin and the trunk musculature is filled with an indifferent fatty connective tissue. In the two specimens examined here there is a difference in this respect between the anterior and posterior parts. Posteriorly the electric tissue ends rather abruptly. A very delicate membrane which is of the same nature as the connective tissue elements within the electric organ separates the electric tissue from the indifferent loose connective tissue situated caudally. The exterior and interior membranes remain separated from each other by this fatty connective tissue, and the posterior part of the body musculature is thus covered all over by a thick coat of connective tissue which forms a caudal continuation of the electric organ.

Anteriorly, the electric organ tapers ventrally and forms a band-shaped structure which reaches the ventral and rostral part of the shoulder girdle (fig. 1). It is of importance that in this region the electric organ, including the exterior as well as the interior connective tissue membranes, is completely independent of the dermal connective tissue, and the space between the dermis and the electric organ is filled up with loose connective tissue of the conventional subdermal type (figs. 2, A and B; 3, A). Fritsch (1887) and many other authors (e.g. Rauther, 1937; Stuart and Kamp, 1934) believed the electric organ to be situated in the skin, separating one exterior and one interior layer of the dermal connective tissue from each other.

The electric organ is distinctly double in the young specimens examined by the present author. There are continuous connective tissue membranes separating the two halves in the dorsal and ventral mid-lines of the body (fig. 3, B). Fritsch considered that these membranes developed gradually during the growth of the individuals and were missing in young specimens. On the other hand, the double nature of the electric organ was assumed by

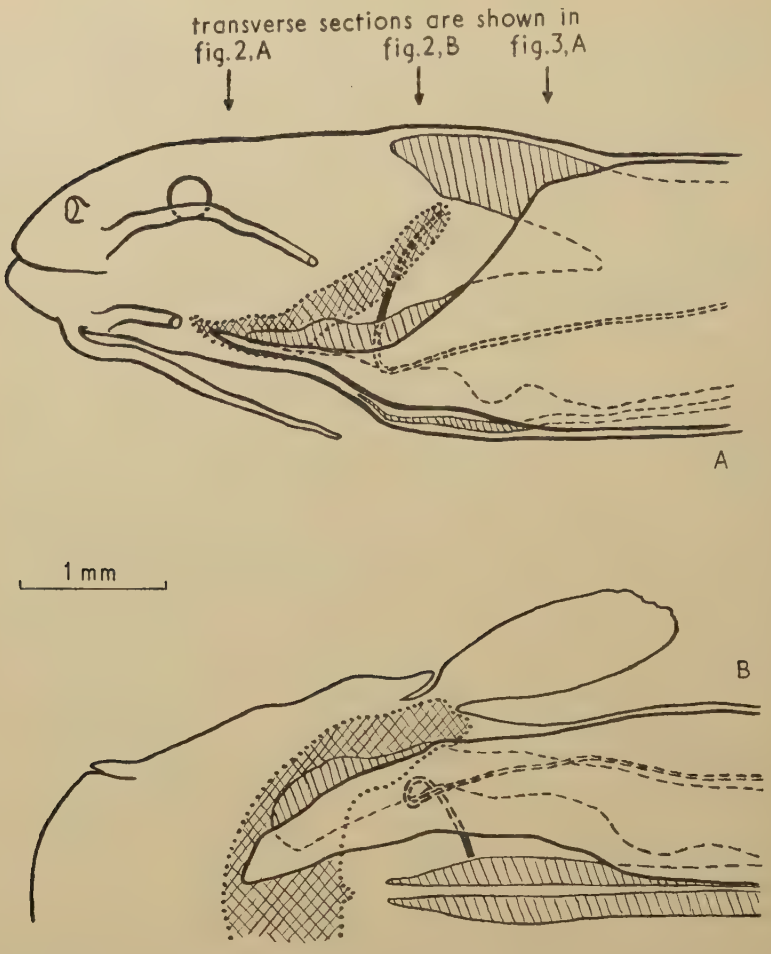

FIG. 1. *M. electricus*, 11·4 mm in standard length. Left portion of anterior part of the body. A, lateral view. B, ventral view. Reconstructions. Thick lines indicate outlines of body and of electric organ. Thin lines and broken lines are contours of trunk-muscles. These are obliquely hatched in the parts not covered by the electric organ. Dotted line indicates the outline of the shoulder girdle, cross-hatched in the parts not covered by electric or muscular tissue. The course of the electric nerve is indicated in the figures. The photomicrographs (figs. 2–4) are from transverse sections of the same specimen.

FIG. 2 (plate). *M. electricus*; photomicrographs of transverse sections. (Positions indicated in fig. 1, A.) A, anterior part of left portion of electric organ (e.o.) close to its attachment to the shoulder girdle (sh.g.). Dorsally to the bone are the truncus arteriosus (t.a.), a rostral portion of the heart (h.), and branchial musculature. Ventrally in the picture is the epithelium (e.). B, section situated caudally to that figured in A. There is a lobation of the growing tissue of the electric organ (e.o.). The electric nerve (n.), artery, and vein are seen at the ventral end of the musculus obliquus. To the right is the liver. There is a wide space between the electric organ and the skin (e.) filled by a connective tissue with very delicate fibrous elements.

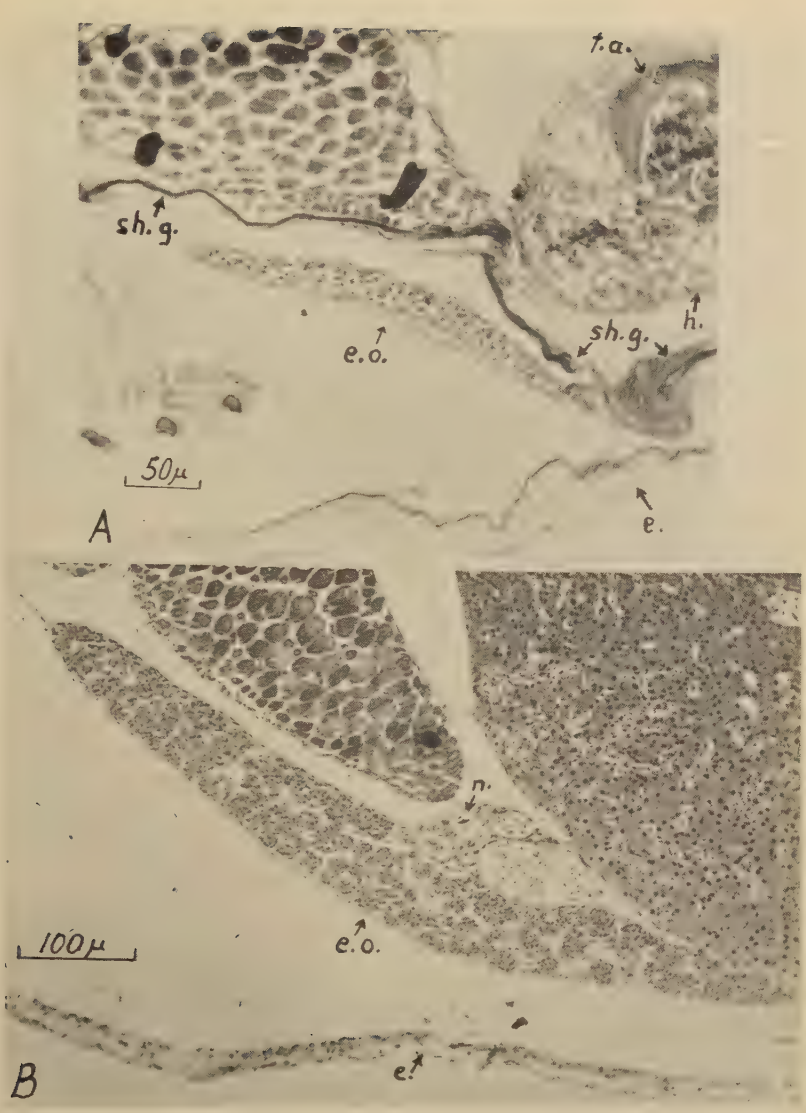

FIG. 2

A. G. JOHNELS

Ballowitz (1899) because of the course of the spinal nerves, which reach the surface of the body only in the dorsal and ventral connective tissue membranes (see also Stuart and Kamp, 1934).

There is a very thin connective tissue membrane which seems to serve to fix the anterior and dorsal edge of the electric organ to the posterior bony rim of the shoulder girdle (seen in the upper left corner of fig. 2, B). No connexion with the axial region dorsally and caudally to the base of the pectoral fin (the position of the axial gland in other silurids) has been observed, nor does any trace of such a connexion exist.

In the small specimens examined by the present author the histological structure of the posterior and middle parts of the electric organ agrees in most respects with the descriptions given in the literature regarding larger specimens. The electroplaxes, although very small, are formed in these portions and the organ seems to be functional there. In the anterior end, on the other hand, the histological structure and the shape of the organ are different from those of larger specimens. In adult specimens the electric organ comes dorsally very close to the posterior part of the head, covering the epibranchial trunk musculature almost completely. There is thus a part of the organ which is situated dorsally and anteriorly to the pectoral fin and the shoulder girdle. This dorsal part of the electric organ is missing in the two young specimens here described (fig. 1). Ventrally the electric organ extends anteriorly as a band-shaped structure to the rostral and ventral portion of the shoulder girdle (fig. 1). In the most rostral portion the exterior and interior connective tissue membranes of the electric organ join each other and are attached anteriorly to the ventral surface of the bone of the shoulder girdle slightly laterally to the ventral midline. The nature of this connective tissue attachment seems to be identical in every respect with a normal muscular tendon, and the electric organ is thus firmly fixed to the bone in this place. The place of attachment of the electric organ to the shoulder girdle (figs. 1; 2, A) is located anteriorly and ventrally to that of the musculus obliquus internus (Maurer, 1913). From this point the electric organ extends caudally in a superficial position to the musculature, between this and the skin (figs. 2 A and B; 3 A and B). Medially, the musculus rectus intermedius and m. rectus medialis (see Nishi, 1938) are attached to a median ligament which is fastened to the small caudal bony projection which is situated on the shoulder girdle (fig. 1, B). In the specimens examined these muscles do not reach the shoulder girdle but are connected with the skeleton by means of tendons. In this way a fairly wide space is formed between the lateral m. obliquus and the medial m. rectus (fig. 1, B). In adult specimens the m. rectus medialis is said to have attained an immediate connexion with the skeleton (Maurer, 1913). As already mentioned, Maurer noticed that there seems to be a small deficiency in the muscular wall in this region of adult *Malapterurus* as compared with other fishes. This deficiency is more obvious in the small specimens examined here.

The electric organ covers the deficiency in the muscular wall exteriorly and in this place the electric nerve reaches the medial side of the organ (fig. 1).

The electric nerve, consisting of one single giant axon with thick connective tissue covers (figs. 3, C; 4, B), originates in the anterior part of the spinal cord (Bilharz, 1857). In adult specimens the giant electric cell is said by Stuart and Kamp (1934) to be situated in the ventral horn of the third segment of the spinal cord. The pictures published by these authors may cause some doubt as to whether the cells observed by them really were the electric cells. These authors were unable, however, to follow the course of the axon, but thought that it most probably emerged in a ventral root. According to Stendell (1915) the electric nerve emerges with the second ventral root. In the present material the electric cell is so large in relation to the diameter of the spinal cord (fig. 3, C) that it is impossible to decide to which of the motor columns it originally belonged, the somatic or the visceral. The statement by Ihle and others (1927) that it is a viscero-motor cell seems to be founded only on the belief that the electric organ in *Malapterurus* has an adenoid origin. The giant electric cell is richly supplied with blood capillaries (fig. 3, C). Several of these penetrate the surface of the cell and are thus intracellular, reminiscent of those of neurosecretory cells (see Scharrer and Scharrer 1954). From the electric cell the axon runs ventrally, medially to the giant fibre of Mauthner, and the electric axon seems to be thicker than this giant fibre (fig. 3, C). The axon emerges in the third of three closely located ventral roots, the anterior of which is considerably smaller than the other two. The space between the third and the fourth spinal nerves is at least twice as large as that between the second and the third. The second and third spinal ganglia are situated very close together, and the ventral rami of the third and second spinal nerves are situated very close to each other just outside the ganglia. In this location, the two nerves are for some distance separated by the electric nerve only. This, although formed by one single axon, is about twice as thick as the ventral rami, owing to the thick connective tissue sheaths. In spite of this close relation it is evident in the sections that there is also peripherally a more intimate connexion between the third spinal and electric nerves than between the latter and the second spinal nerve. After a short distance the three nerves separate, first the ventral ramus of the second from the other two and a little farther away that of the third spinal nerve from the electric nerve.

The tissue between the connective tissue membranes of the most rostral

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FIG. 3 (plate). *M. electricus*. A, photomicrograph of transverse section of anterior portion of electric organ (e.o., position of section indicated in fig. 1, A). The arrangement of forming electroplaxes is seen in the dorsal part, adjacent to the electric nerve (n.), artery, and vein. The space between the skin (e.) and the electric organ is artificially enlarged in the dorsal part of the picture by a rupture in the intermediate connective tissue. The normal space is seen in lower part of the figure. B, transverse section through middle portion of electric organ. Photomicrograph. C, photomicrograph of transverse section through the anterior part of spinal cord showing the right electric nerve-cell. The axon is seen to emerge from the electric cell, running medially to the giant fibre of Mauthner (M.). Ventrally it ends, in the section, in the middle of the third ventral spinal root (3 v.). The electric nerve (n.), lined with the fibres of the ventral ramus medially, dorsally, and laterally, is seen to the right outside the canalis spinalis, ventrally to the spinal ganglion (s.g.). The positions of some of the intracellular capillaries of the electric cell are indicated by arrows.

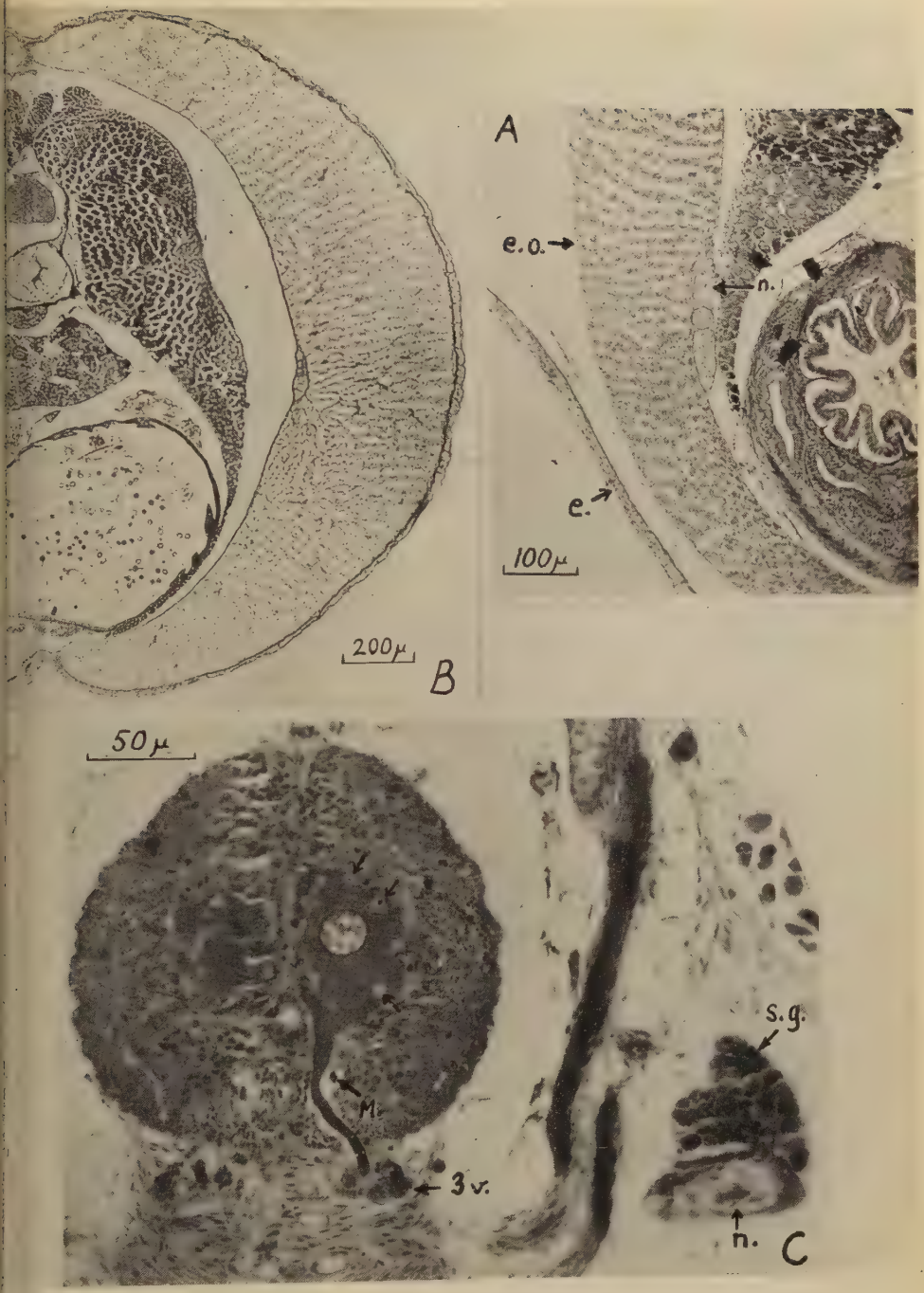

FIG. 3

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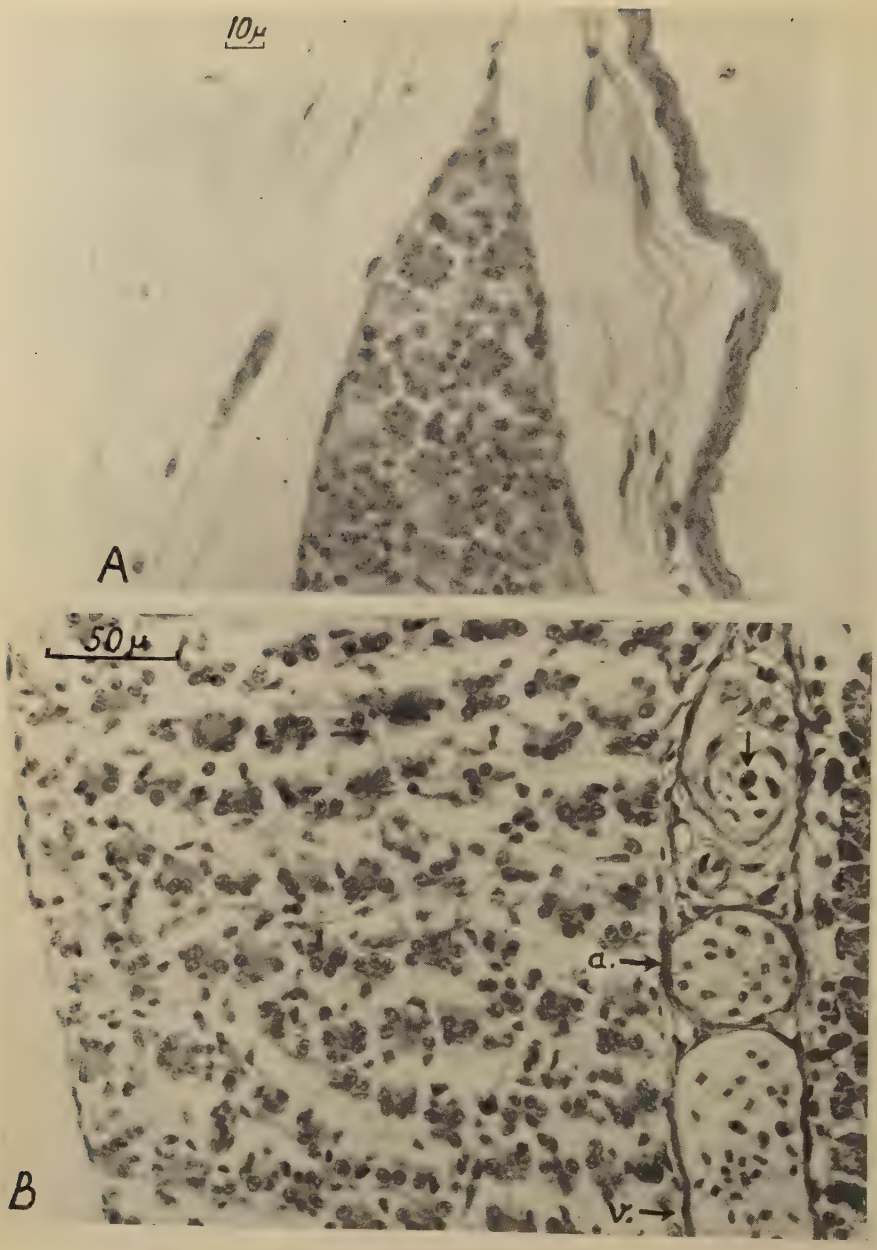

FIG. 4

A. G. JOHNELS

portion of the electric organ consists of large numbers of nuclei very sparsely surrounded with cytoplasm (fig. 2, A). There is a certain lobation of the cell masses by connective tissue trabeculae formed by very delicate elements, particularly evident a little more posteriorly (fig. 2, B). In the region on a level with the entrance of the electric nerve and particularly in the part of the organ which is situated just caudally to this place there is a formation of electroplaxes (fig. 3, A). This formation is completed gradually in a caudal direction. The cells group themselves together in small circles, mostly 6 to 8 in number. These cells start forming a small syncytial mass of cytoplasm between themselves in the centre (fig. 4, A and B). This cytoplasm appears blue in the preparations and develops into a small disk the periphery of which is lined with the nuclei of the cells mentioned. In this anterior region of the organ mitoses are numerous. In the middle and caudal portions of the electric organ no mitoses have been observed. As is well known, the postembryonic growth of the organ is caused by an increase in size of the electric plates. There is no increase in number of the electroplaxes. The anterior region of the electric organ of the small specimens examined here is thus the centre of formation of electric elements.

The forming electroplaxes are arranged in fairly regular lines which are orientated approximately at right angles to the interior and exterior connective tissue membranes of the electric organ (figs. 3, A; 4, B). This regular arrangement is retained also by the more fully developed electroplaxes in the middle and caudal portions of the organ (fig. 3, B). There is thus at least originally a regular arrangement of the electroplaxes in *Malapterurus*. However, there is no formation of piles of electric plates in *Malapterurus* as in other electric fishes.

Discussion

Ballowitz (1899) considered that the similarity in histological structure between the electroplax in *Malapterurus* and that of other electric fishes was a reason for assuming that the source of origin is the same in all electric fishes, namely, the striated musculature. On the other hand, it has been argued that a similar structure may be caused to arise because of a similar function in spite of a different source of origin (see Rauther, 1937). In any case the idea of an adenoid origin of the electric organ in *Malapterurus* gained a majority of supporters, although real evidence in favour of this alternative seems to be as scanty as in favour of the other.

As already mentioned, Maurer (1913) observed a small deficiency in the muscular wall (this deficiency, as a matter of fact, can be seen in a figure

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Fig. 4 (plate). *M. electricus*. A, the formation of electroplaxes. Transverse section of the dorsal margin of the electric organ. The connective tissue membrane lining the body cavity to the right. A wide space with loose connective tissue separates the organ from the dermal layer of the skin (not seen in the picture). B, transverse section of the electric organ with forming electroplaxes and electric nerve (axon indicated by an arrow), artery (a.), and vein (v.). Body musculature to the right.

published by Bilharz in 1857, although nobody then stressed the point) in the region adjacent to the place where the electric nerve joins the electric organ. However, Maurer's observation received no attention. On the contrary, it was later stated in the literature that there was no sign of any deficiency that might indicate a muscular origin of the electric organ (Rauther, 1937).

The electric nerve is motor, a fact that suits both ideas. The fact that it emerges through a ventral root cannot exclude the possibility that it is visceral. That this nerve consists of only one giant nerve-fibre which supplies all electroplaxes in one of the halves of the electric organ may merely denote that a very small original primordium is responsible for the subsequent enormous development of the organ, but this tells nothing as regards the nature of this primordium. If the source of the organ is muscular this primordium may be so small that it belongs to one single segment, and this fact may explain why there is no trace of regular piles in the electric organ of *Malapterurus* as is the case in other electric fishes. On the other hand, the possibility of a process of concentration of an originally more complex innervation is not excluded as far as the structure of the nerve is concerned.

The present material permits the following statements. (1) The formation of electroplaxes of the electric organ in *Malapterurus* takes place in the rostral part exclusively. This evidence strongly supports the conclusion that the original primordium is to be found in this region. (2) In the same region there is a distinct deficiency in the muscular body-wall and here the electric nerve reaches the electric organ. (3) The anterior portion of the electric organ, representing the centre of growth, replaces closely in position the missing parts of the musculature, and (4) is fixed by means of a structure identical with a muscular tendon to the very part of the shoulder girdle where the missing muscles ought to have been inserted, if developed. (5) In the region of growth the electric organ is completely independent of the skin and not only so of the epidermis but also of the dermal connective tissue. This definitely speaks against the idea of an adenoid origin. Further, there is nothing in the histological picture which speaks against the possibility that the origin of the cells of the growing portion may be myoblasts, although this is not particularly indicated in the present material.

To a large extent the evidence combines to indicate that the organ has evolved from muscular tissue. There is no evidence inconsistent with this conception, and nothing has been observed in the present investigation that might support the idea of an adenoid origin of the electric organ in *Malapterurus*. The conclusion is, thus, that the electric organ in *Malapterurus* is of myoblastic origin as in all other electric fishes which have so far been studied in this respect.

The arrangement of regular piles of electroplaxes which is found in other electric fishes probably exhibits something in common with the original structure of the electric tissue, the striated muscles. This similarity is lacking in *Malapterurus*. This may indicate that the electric organ of the electric catfish is more specialized than in the other electric fishes. Also the fact that there

is no formation of structures similar to vestigial muscle fibres during the ontogeny of the electric organ in *Malapterurus* is consistent with this idea.

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